HIV PREVENTION TRIALS NETWORK
MICROBICIDE TRIALS NETWORK
LABORATORY MANUAL

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HIV PREVENTION TRIALS NETWORK/MICROBICIDE TRIALS NETWORK

LABORATORY MANUAL

Section 1: Introduction, Quality Assessment and Quality Control

Who are we?

HIV Prevention Trials Network (HPTN) is a worldwide collaborative clinical trials network that develops and tests the safety and efficacy of primarily non-vaccine interventions designed to prevent the transmission of HIV. The HPTN has proactively addressed its goal of developing a state-of-the-art, collaborative, multi-site, multi-trial, multidisciplinary prevention science research agenda across five scientific disciplines included in its mandate from NIH; antiretroviral, behavioral, perinatal, STD control, and substance use.

Microbicide Trials Network (MTN) is a worldwide collaborative clinical trials network that evaluates the safety and efficacy of microbicides designed to prevent HIV transmission. The mission of the MTN is to reduce the sexual transmission of HIV through the evaluation of microbicide products. The MTN will conduct scientifically rigorous and ethically sound clinical trials that will support licensure of topical microbicide products.

Scope of the HPTN/MTN Laboratory Manual

All HPTN and MTN study sites are required to adhere to Good Clinical Laboratory Practices (GCLP) as described in this document, Network Manuals of Operations (MOP), Study Specific Procedure manuals (SSP), NIH/NIAID/DAIDS standards and any applicable local regulations.

This manual provides information pertaining to laboratory tests and issues which are frequently involved in HPTN/MTN studies. Also included are sample SOP's; these SOP's are to be used as examples to model local SOP's after adaptation to site conditions and approval of contents. This manual does not cover every aspect of laboratory science and will need to be supplemented with information from operator's manuals, package inserts, textbooks, reference materials, journals, etc. A list of useful references is included at the end of this manual.

- *GCLP*
  Good Clinical Laboratory Practices (GCLP) is a set of standards designed to ensure that accurate and repeatable laboratory results are generated. It can be generally subdivided into the following subjects: organization and personnel, facilities and equipment, testing facilities operations, test and control articles, records and reports (documentation). Many of these issues are covered in this document.

- *Quality Assessment and Quality Control*
  Quality Assessment (QA) is the systematic activities implemented within the laboratory to ensure that laboratory results are as accurate and reliable as possible. Quality Control (QC), an element of quality assurance, is an essential component of every lab test. QC in the laboratory contexts refers to the specific methods for determining the acceptability of individual test procedures. Each HPTN/MTN laboratory will need to have its own QA Plan and Procedures. This document will address some of the important issues of QA/QC. Further information will be found in network MOP's.

We appreciate your feedback on this manual. If you have any questions or comments please contact either the HPTN or MTN Network Laboratories at

CentralLab@HPTN.org mtncentrallab@mtnstopshiv.org

Procedure: Quality Assurance Policy Version 5 Quality Assessment Policy Version 3

Prepared by	**Date Adopted**	**Supersedes Procedure #**
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Review Date	**Revision Date**	**Signature**

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PURPOSE

The laboratory has an ongoing Quality Assessment Program that is designed to monitor, evaluate and improve the quality of laboratory performance. This program also ensures the reliability of test data and the competency of the laboratory staff. The lab will identify and resolve any problems that may affect lab performance and thus patient care.

For the purpose of this document, any work area in which testing of patient samples occurs is subject to the same sets of guidelines and policies as the laboratory. This includes clinic areas and off site locations. Any individual who performs testing on patient samples must adhere to the contents of this policy.

Additional quality assurance procedures may also be listed in Study Specific Procedure manuals.

Manufacturer recommendations must be followed. If the documentation the lab receives gives conflicting information, please contact the HPTN Central Lab for advice. (CentralLab@HPTN.org)

OBJECTIVES

1. To ensure that the quality assurance activities are comprehensive and coordinated and that appropriate information is reviewed and reported.
2. To establish, maintain, support, and document an ongoing Quality Assurance Program that includes effective and systematic mechanisms for monitoring, collecting and evaluating information about important aspects of laboratory performance in order to identify opportunities for improving patient care.
3. To assist in improving care and identifying problems through the use of ongoing monitors by focusing on identification, assessment, correction, and follow-up problems that affect laboratory performance.
4. To implement corrective action when problems or opportunities are identified.
5. To follow up on identified problems to assure improvement and resolution in a timely manner with documentation of corrective action.

QUALITY ASSURANCE MONITORS

The following Quality Assurance monitors are actively evaluated to maintain an established standard of laboratory performance and compliance. Data from each monitored area is collected, recorded and analyzed. The findings are evaluated to detect trends and overall compliance. When required, appropriate corrective action will be implemented and documented. Monitoring will be continued to assure that the action taken was appropriate and resulted in correction of any problems found. At a minimum, quarterly meetings are held to review the monitored area reports.

Proficiency Testing:

Please also refer to the Instructions for handling CAP Proficiency Surveys Guidelines.

Proficiency programs are used as an external check on the quality control and quality assurance of a test system. Analytes should be tested a minimum of twice per year, with the recommendation being 3 times per year.

The laboratory will participate in external proficiency panels/surveys, which are blind assessments of the laboratory's performance. Where possible, the laboratory will participate in a proficiency program for each test performed in the lab/clinic area
For testing where no external proficiency program samples are available, other methods of proficiency checks will be used..

1. Proficiency samples are tested in the same manner as any routine specimen submitted to the laboratory.
2. No special assignments will be made for the testing technologist; the routine bench schedule will be followed.
3. The laboratory supervisor or designee will review the final result forms and send to the testing agency in a timely manner.
4. A copy of the final results form will be kept in the External Proficiency Testing file.
5. When the survey results are returned, the laboratory supervisor and director will review and sign the results.
6. If there are any noted deficiencies, the deficiencies will be investigated by the laboratory supervisor and director. The DAIDS contracted group will send a PRF form to the laboratory on all failed or missed analytes.
7. A written report of the findings and corrective action will be written. The laboratory supervisor and director will sign this report. The PRF form should be completed.
8. The report will be sent to the DAIDS contracted group for review.
9. A copy of the response will be filed with the survey results.

All proficiency program reports should be reviewed, signed and dated by the laboratory supervisor and director as soon as possible upon receipt. The signed copy should be filed with the original results. The laboratory supervisor and director must review any deficiencies cited by any proficiency program or accrediting organization in which the laboratory participates.

1. The director, or designee, must submit in writing a plan of corrective action within two weeks of notification of any deficiencies to DAIDS contracted group as well as the HPTN Network Laboratory (centrallab@hptn.org) and/or MTN Network Lab (mtncentrallab@mtnstopshiv.org). For IQA or VQA proficiency panels, please submit the corrective action plan to the appropriate contact person for those agencies as well as the DAIDS contracted group as well as the HPTN Network Laboratory (centrallab@hptn.org) and/or MTN Network Lab.
2. The deficiency report will include an explanation of the likely cause(s) of the deficiency along with appropriate corrective action, if indicated.
3. These deficiency reports will be filed in the proficiency test result manual with the original report.

Specimen Management:

Specimens sent to the lab are monitored to determine the effectiveness of the collection procedures as well as the integrity of the specimens received. The following areas will be monitored, recorded and investigated in a timely manner:

1. Lost specimens (at point of collection to lab or within the lab).
2. Rejected specimens (unsuitable specimens).
3. Missed Testing – test missed by lab.
4. Specimen integrity – specimen too old to test or stored at wrong temperature

Reporting of Results:

Results released to the clinician or study personnel are monitored to determine the effectiveness of the laboratory review and reporting system. The following areas are used to monitor the accuracy of released results.

1. A portion of the results (not less than 5%) released from the laboratory are to be monitored by comparing the final report result to the result on the worksheet. This review is to be documented along with any discrepancies detected and the corrective action taken.
2. The number of modified or amended results is to be documented along with the reason for the change and any corrective action taken.
3. The laboratory must have a policy in place to deal with the reporting of amended results.

Technical Delays:

Technical delays are monitored to help evaluate the overall effectiveness of the laboratory. Any time delay in reporting of patient test results due to a technical problem in the lab needs to be documented. This includes such parameters as scheduled and unscheduled instrument down times, acute or chronic staff shortages, contaminated cultures, failed reagents, failed quality control, and supply back orders. Clinic staff need to be notified when the downtime causes delays in resulting of routine reports if the delay is to exceed the established turn round time. If the delay will adversely affect the study, the laboratory should discuss the issue with the clinic staff and the HPTN Network Laboratory and/or MTN Network Laboratory to determine if the back-up plan needs to be implemented.

Turn around time (TAT) is a measurement of technical delays and it can be affected by items such as specimen transport difficulties or the above mentioned technical problems in the laboratory. Maximum acceptable turn round times must be available to the laboratories clients. The Laboratory Director mandates the TAT for each test. The monitoring of pre-analytical, analytical and post-analytical processes help to identify potential problematic areas within the laboratory. TAT should be reported to clientele on a periodic basis. Recommendation is monthly.

Complaints:

Complaints received by the laboratory are monitored for response, corrective action and follow-up. The supervisor or designee will respond to any written or significant oral complaint concerning the quality of service or results. Patient care/well being as well as clinical study support are taken into consideration in designing and responding to the corrective action. The timeline for responding to complaints needs to be defined by the laboratory. Responses to complaints will be forwarded to the director for review and any additional recommendations of appropriate action

Performance Improvement Monitoring/ QIP – Quality Improvement Program

The laboratory will identify potential problems or areas of improvement within the laboratory. These areas will be monitored for problem frequency, possible causes, corrective action and improvement. The information will be documented by the laboratory supervisor or designee and reviewed by the laboratory director.

TRAINING

New Employee: Lab specific job descriptions entailing the duties of each employee are kept on hand in the individual personnel files. Each employee must read and sign off on their particular job description. A checklist for the training of new personnel should be established for the assays in the laboratory. Personnel sign each section on the checklist as well as their trainers. These records are kept in the personnel file and should be available for inspection. Please note that evidence of training is not evidence of competency.

New Procedures/New Equipment:
Each employee must be trained on new procedures or new equipment. The training must be documented and signed by the employee and the trainer. These records are kept in the employee's personnel file and should be available for inspection. Please note that evidence of training is not evidence of competency.

CONTINUING EDUCATION:

Continuing education provides personnel an opportunity to review and expand their knowledge of laboratory procedures and policies, and any other subjects pertinent to successful laboratory operations.

1.Each technical employee is required to fulfill a minimum of 10 hours per year.
2.Continuing education credit may be earned through reading, watching videos, listening to cassette tapes, attending departmental lectures, teleconferences, training seminars, workshops, as well as tech sample reviews or safety training (fire safety, universal precautions, blood borne pathogens).
3.IATA Dangerous Goods Shipping certification is required every 24 months. Review of IATA regulations is required annually.
4.Each employee should keep a record of his or her continuing education. Any supporting documents should be given to the supervisor to maintain in the personnel file. (Refer to appendix for CE Record form.)

QUALITY CONTROL

Each procedure outlines the required control materials and analysis frequency for the tests performed in the laboratory or other testing location. It is the responsibility of every technologist to ensure that the required controls have been performed and satisfactory performance has been obtained prior to the release of any patient results.

Please refer to the Quality Control Policy version 5.0 for further information.

NEW REAGENT LOT VALIDATION.

Reagent kits and controls the lab uses have a limited shelf life. It is important to ensure that test kits and reagents are not used beyond their expiration date. New lot check in of reagents is done in order to validate the lot to lot variability.

1. **HIVEIA Assay**: In order to validate the lot to lot variability with the HIV EIA assay, a minimum of 3 patient samples (negative, low positive and high positive) identified by the lab supervisor are run using the new reagent/kit lot and the in use lot. The patient results should be reproducible between the two lots. The laboratory supervisor or director will sign off on the validity check. The patient samples will be marked validation samples and filled with the other HIV EIA runs.

2. **HIV RNA PCR, Quantitative Assay**: In order to validate the lot to lot variability, three (3) patient samples (not detected, a mid range viral load and a high viral load) are assayed on the old and the new lot number. The laboratory supervisor or director will sign off on the validity check. These results will be recorded in chart form and filed with the quality control records for this assay by the laboratory supervisor. As the laboratory is starting to perform the assay, lot to lot variation should be less than 0.5 log with any variation greater than 0.3 log difference being investigated and documented. After the lab is established this difference may be tightened but the ultimate decision is made by the laboratory director. Please note: commercial standards or those provided through the Virology Quality Assurance (VQA) can be utilized in place of patient samples.

3. **PCR(HIV, GC, Chlamydia) Qualitative Assay**: In order to validate the lot to lot variability, a minimum of 3 patient samples (negative, low positive and high positive) are run using the in-use lot and new lot of reagent/kit. The patient results should be reproducible between the two lots. The laboratory supervisor or director will sign off on the validity check. The patient samples will be marked validation samples and filled with the other PCR runs.

4. **SDA (GC, Chlamydia) Qualitative Assay:** In order to validate the lot to lot variability, the positive and negative control samples from the old lot are assayed on the new lot/kit. The laboratory supervisor or director will sign off on the validity check.

5. **Other ELISA Quantitative Assays**: In order to validate the lot to lot variability with the p24 media assay, a known positive supernatant from a previous run is assayed. The laboratory supervisor or director will sign off on the validity check. The patient samples will be marked validation samples and filled with the other P24 runs

6. **CD4/CD8 Assay**: In order to validate the lot to lot variability of reagents, a minimum of 2 patients (one with CD4/8 ratio <1.0 and one with CD4/8 ratio >1.0) are run using both the in use and new lots of reagents. The patient results should be reproducible (based on manufacturers guidelines for sample to sample, lot to lot variation) between the two lots. Typically the results should be within 15% of each other. The laboratory supervisor or director should sign off on the validity check. The patient samples will be marked validation samples and filled with the other CD4/8 runs. It is also important to check expiration dates and perform lot testing on primary and secondary antibodies used for this purpose. For further information, check the IQA Q & A on the HPTN website.

7. Chemistry, Hematology and Coagulation – New Reagent Lot Check In:

New lot numbers of reagent must be validated before being introduced into routine use. 10 patient samples must be assayed using the old reagent are re assayed using the new reagent. QC should be acceptable for old and new lots. Samples should be assayed by both lots within a time period in which there has been no loss of integrity to the sample or analyte. Results should be compared to the old lot. Acceptability criteria should be set by the Lab Director.

8. Examples of Other Internal Quality Checks

All reagents and consumables must be examined prior to use. As an example:

a.. Culture Media
   1. All culture media will be checked for expiration dates before being put into use.
   2. A culture media control log will be used to document the lot number and quality control results.

3. Any media that appears cloudy, has a color change or shows contamination will be discarded.
4. Appropriate control organisms will be used to check selective media.
5. The control log will be initialed and dated by the technologist performing the quality control.
6. The control log will be reviewed and signed at least once per month by the laboratory supervisor or QA/QC technician/designee.
7. Media that fails the quality control check will be documented and discarded.

b. Animal Sera
   1) All animal sera will be checked for cytotoxicity before use.
   2) An animal sera control log will be used to document the lot number, expiration date and quality control results.
   3) Acceptance criteria for cytotoxicity will be defined.
   4) The control log will be initialed and dated by the technologist performing the quality control.
   5) The control log will be reviewed and signed at least once per month by the laboratory supervisor or QA/QC technician/designee.
   6) Animal sera that fails the cytotoxicity check will be documented and discarded.

VALIDATION STUDIES

Any time an instrument or methodology is changed within the laboratory, or if the testing location is changed, validation studies must be performed. Analyzers shipped for repair must undergo re-validation on their return prior to being reintroduced into routine use. Borrowed equipment (loaners) must also undergo validation studies before being put into use.

Please Refer to HPTN Quantitative and Qualitative Assay Validation Policies for details.

New analyzers or assays should not be put into place until the necessary data and documentation has been approved by the Network Lab.

Analyzers and assays should not be changed mid trial. If an assay or analyzer has to be changed mid trial, do not do so until the necessary data and documentation has been approved by the Network Lab. Changes should also be proposed to the protocol team before being made.

METHOD COMPARISON

Performed semi-annually between similar instruments or methods. Run a minimum of 10 samples and perform comparison.

There must be a back up method available for protocol related safety and end point assays. This can be performed in-house or at a back up lab. Primary methodology and

back up methodology must be compared during initial validation and semi annually thereafter.

The lab director sets the acceptable limits of the method comparison.

CARRYOVER

Sample carryover may cause one high patient sample to affect the sample that follows it. Most of today's diagnostic analyzers take every possible precaution to avoid sample carryover. In spite of these efforts a sample having a high result may affect one or more samples that follow it. The lab must show that their instruments or test system does not have any unacceptable carryover.

Follow manufacturer instructions for assessing carryover and acceptability limits.

Carryover studies must be performed during assay validation , and when carryover is suspected.

PROCEDURE REVIEW

All procedures used in the lab must be documented and reviewed.

1. All laboratory procedures are reviewed on an annual or more frequently if needed basis.
2. Procedure reviews are done by the supervisor and director on an annual basis. Any changes that occur at that time need to be communicated with the staff. Any changes that occur prior to the annual review must also be communicated with the staff. Note: GCLP training has suggested havingan annual review be the same time for all procedures. In some cases, it may be advisable to have a monthly schedule of procedural reviews.
3. Each procedure is preceded by the documentation of review (also known as a signature page).
4. Modifications of a procedure can occur at any time due to newly published guidelines, revised package inserts, changes in central policy, etc. All revisions should be documented in ink on the original copy along with initials of the supervisor or designee and the date of change. This superceded/obsolete copy must be kept for at least 5 years or indefinitely if the procedure was involved in a DAIDS trial..
5. The revised procedure should include the revision number and effective date to help identify it as the current procedure.
6. All changes must be documented and communicated to the technical staff. Retraining and competency check of the staff must occur for any procedural changes.
7. Appropriate version control must be maintained.
8. Any copies of procedures must be pulled and replaced with the updated version.
9. Documentation for all HPTN/MTN protocol related procedures must be approved by the HPTN/MTNNetwork Laboratory prior to study activation.

COMPETENCY:

New employees are checked for competency twice during their first year of employment in the laboratory. The first competency check should be completed before any patient results are reported by the new employee. Existing employees are checked annually and periodically as needed. Competency may be checked by:

1. Direct observation (use SOP or a check list to insure no steps are omitted)
2. Quality control result review
3. Repeat and split sample testing
4. Unusual patient or unusual control result review
5. Proficiency testing review
6. Blind specimen analysis.
7. Oral examinations

If an employee fails his/her competency checks, they must complete a retraining procedure and pass a further competency evaluation before they can test patient samples

Blind or Split Sample Testing (Internal Proficiency Testing)

1. As part of the laboratory's internal proficiency testing program, personnel proficiency testing is done periodically during the year. Coded samples, blind samples or split samples may be given to the technologists and or clinic staff to assess the reproducibility of the assays as well as the technologist to technologist variability and accuracy.
2. The laboratory supervisor or designee (such as the QA/QC technologist) will be responsible for assigning the samples, documenting the results and reviewing the results.
3. The acceptable range of reproducibility will be determined by test and documented on the result form.
4. The documentation will include the results by technologist and whether the results compared acceptability for accuracy and reproducibility.
5. The laboratory supervisor and or director will sign off the results.
6. The results will be filed as Internal Proficiency Testing records.

STORAGE OF LABORATORY RECORDS:

All laboratory records inclusive of requisitions, patient results, QC logs, maintenance logs, QA logs are retained for indefinitely. DAIDS will notify the lab when records can be destroyed. Records are to be stored in an orderly manner that allows retrieval within 24 hours. Records may be stored off site and on site in locked and secure storage.

RESULT MODIFICATION / AMMENDMENT;

In the event of incorrect verification of data, the incorrect result needs to be modified and the correct result entered. Discrepancies are to be resolved immediately.

1. All modified results must be brought to the attention of the ordering physician/clinic and documented.
2. The modified report must include the initials of the lab supervisor as well as a brief explanation if appropriate. The original result should stay on the patient chart with a note that it has been amended.
3. Modified (amended) reports will be documented under the quality assurance monitoring.

RESULT REPORTING CHANGE:

Changes in test methodology and /or reference ranges must be communicated to the ordering staff by a lab note or department memo. These changes should be communicated to the HPTN/MTN NL before implementation. These changes must also be communicated to the FHI CRM and SDMC POC associated with the study as changes may have an effect on data analysis or safety reporting requirements.
Changes in methodology and / or reference ranges for HPTN/MTN protocol assays must be approved by the HPTN/MTN Network Laboratory.

MAINTENANCE OF EQUIPMENT;

A separate manual for equipment maintenance is kept in the laboratory. Each instrument in use has a separate procedure for maintenance and the time frame for the performance of the maintenance

Maintenance of equipment should follow manufacturer recommendations at a minimum.

1. Maintenance log sheets are kept on a daily, monthly, quarterly, semi-annually, and annually basis.
2. These records are reviewed and signed by the laboratory supervisor or director.
3. These records are retained for a minimum of five years.
4. Any preventive maintenance, repairs, or part replacement records is kept for the lifespan of the equipment or 5 years (whichever is greater).

Instrument Maintenance

1. All instruments used in the lab follow a preventative maintenance program which must follow the manufacturer's recommendations.
2. Documentation of the instrument maintenance, calibration, service and corrective action logs is generally found in the bench workbook for each area.
3. The area technologist maintains these records.
4. These records are reviewed and signed monthly by the laboratory supervisor or designee.

Equipment Maintenance

1. Routine maintenance on laboratory equipment is performed according to the manufacturer's recommendations.
2. The technologist performing the maintenance documents the maintenance and results.
3. The laboratory supervisor reviews and signs off the maintenance records monthly.
4. Documentation of the equipment maintenance is generally found in the laboratory Maintenance Manual.
5. Preventative maintenance, monitoring or calibration generally covers the following equipment:
    a. Precision pipette calibration
    b. Centrifuge calibration (rpm, timer and temperature if applicable)
    c. Thermometers
    d. Timers
    e. Plate washers
    f. Plate readers
    g. Thermocyclers
    h. Incubators
    i. Biological/Fume Hoods

Temperature Monitoring (include a chart to monitor room temperature for labs)

1. All temperature sensitive equipment such as freezers, refrigerators, water baths and incubators must be monitored on a regular basis. ie. at least each working day.
2. All test work areas and reagent storage areas must be monitored on a regular basis. ie. at least each working day. (ie room temperature monitoring where equipment and testing is done as well as where room temperature reagents are stored).
3. Temperature charts must include the name of the equipment (if applicable), the location, the acceptable temperature range, space to record the actual temperature and the initials of the person recording the temperature.
4. The temperature chart may include a comments/corrective action section or corrective action may be recorded on another form.
5. The charts must be reviewed on a monthly basis by the laboratory supervisor.

Reagent Water

The following procedures and specifications are for the testing of water which has been purified for clinical laboratory use. There are three grades of water recognized along with the minimum specifications for bacterial count for each.

Type I	Used for the preparations of solutions, reagents (EIA testing) requiring minimum interference and maximum precision and accuracy (10cfu/ml)
Type II	Used for general laboratory testing other than described above
Type III	Used for glassware washing, but not final rinsing and for feedwater for production of higher-grade water

The preferred water is Type I, distilled, deionized water. If this is not available, distilled water can be used and sterilized if necessary.

If the laboratory has a water purification system, the quality of the water must be checked on a regular basis. ie. at least each working day. This must be documented on a chart which may include a comments/corrective action section or corrective action may be recorded on another form.
The charts must be reviewed on a monthly basis by the laboratory supervisor.

LDMS Reconciliations

1. On a weekly basis, the laboratory and the clinic must reconcile the samples sent by the clinic for each protocol with what the laboratory has reported as received. Any protocol related issues (insufficient sample, too few aliquots) should be brought to the attention of the HPTN/MTN NL as well as the appropriate FHI and SCHARP staff.

2. In addition to the weekly reconciliations, SCHARP staff will be sending site specific reports on the SCHARP reconciliations between the exported LDMS data and the data-faxed CRFs. The laboratory must report any corrective actions to the HPTN/MTN NL within two weeks of receipt. Data Modifications should be performed following the FSTRF document. Any PTID changes must be approved with the appropriate NL.

Correcting Specimen Records in the LDMS

This document lists the procedure of how specimen records should be corrected in the LDMS and how changes should be communicated if the specimen has been shipped to another lab.

Specimen Management

Please follow the steps listed below to correct a specimen record in the Specimen Management Entry Tab:

1. Correct the appropriate field (i.e. modify the specimen date).
2. In the primary grid click on the details button.

3. In the details dialog box enter the following information in the comments field: Name of the field changed, previous and current field values, initials of who made the change and date the correction was made.
   Example: Specimen Date changed from 04/01/2002 to 04/02/2002 on 04/19/02 by JES.
4. Once the information has been entered in the comments field, click the OK button on the specimen details dialog box.
5. Click the OK button on the warning message box (Warning: Comments will be shipped. If you do not want your comments to be shipped please enter as user defined ID's.).
6. Click the Yes button on the warning message box (Do you wish to cascade this primary's comments to its aliquots?).
7. Click the Save button on the toolbar, to save the modifications made to the patient record.
8. Click the OK button on the Save dialog box that appears.
9. A label dialog box will appear asking if you would like to generate labels. If you want to display labels for the aliquots you just added, select the label size from the list and click on the Yes button. Note: Labels can also be generated in the labels module.
10. If possible, relabel the aliquot(s) or Dried Blood Spot with the corrected label.
11. If the sample was shipped to another lab, the lab must notify the receiving lab of the changes made in the LDMS by emailing or faxing a copy of the Specimen Log Report (see Reports section below). To ensure consistency, the receiving lab must also make the same corrections in the LDMS for the sample.

Reports

For specimens that have been shipped, please follow the steps listed below to email or fax a copy of the Specimen Log Report:

1. To access the Specimen Log Report, click on the Report button on the LDMS toolbar.
2. Select "Specimen" from the Category grid and "Specimen Log Report" from the Description grid.
3. Use the selection criteria fields to define the records that should appear on the report. See Chapter 6 of the LDMS User Manual to define your criteria statement.
4. Once the criteria have been selected, click the Execute button on the LDMS toolbar.
5. If **faxing** the report to the testing lab, click the Print button on the report toolbar and then click the OK button on the Print dialog box that appears.

   OR

   If **emailing** the report to the testing lab, click the Export button on the report toolbar. Select a format from the Export dialog box that appears (Word format is suggested) and click the OK button. Select the drive and or folder on your computer hard drive into which you would like to save the report and click the save button. Attach the file to the appropriate email.

In addition to the above mentioned checks, each laboratory needs to perform self-audit checks of their LDMS storage. The recommendation is weekly. Storage reports should be printed and the physical locations reviewed. NL staff will also perform these checks when on site.

Local Laboratory Monitoring

The DAIDS Clinical Site Monitoring Group (PPD) conducts quarterly monitoring visits to HPTN study sites with ongoing studies (see also Section 16 of the HPTN Manual of Operations). In addition to performing monitoring tasks specified by the Division of AIDS (DAIDS) in study clinics and administrative locations, monitors also will perform monitoring tasks specified by DAIDS in each site's local laboratory or laboratories. Laboratory monitoring tasks may include inspection of laboratory facilities and documentation as well as confirmation of the use of LDMS and verification of specimen storage as recorded in LDMS. Specimens selected for on-site verification generally will not be pre-announced to site staff.

Procedure: Quality Control Policy Version 5.0

Prepared by	**Date Adopted**	**Supersedes Procedure #**
Estelle Piwowar-Manning		Version 4.0
Sarah Dawson		
Paul Richardson		

Review Date	**Revision Date**	**Signature**

Distributed to	**# of Copies**	**Distributed to**	**# of Copies**
HPTN Laboratories			

PRINCIPLE

Quality Control is an important part of every laboratory test. Appropriate Quality Control practices will maximize the accuracy of results reported as well as possibly provide early information of potential problems. This procedure is intended to give a summary of the quality control program to be followed in the laboratory. A detailed description of the quality control procedures for individual assays should also be included in the quality control sections of the individual procedures.

The laboratory recognizes that the institution and maintenance of a rigorous quality control program can assure the reliability of patient laboratory data. As the spectrum of the tests offered is broad, so are the quality control procedures and the way in which data from various types of quality control material are handled and presented.

This policy applies to any testing procedure associated with the HPTN. This includes Laboratories, clinic areas and other off site testing areas.

Additional quality control procedures may also be listed in Study Specific Procedure manuals.
Manufacturer recommendations must be followed. If the various documentations gives conflicting information, please contact the HPTN Network Laboratory (CentralLab@HPTN.org) for advice.

Laboratory Internal Quality Control Requirements:

1. Laboratory Internal Quality Control material must be analyzed on a regular basis at least according to manufacturer recommendations.
2. QC Data for protocol related assays for hematology, chemistry, flow cytometry and coagulation must be submitted on a monthly basis to HPTN Network Laboratory, attention Domestic Quality Assurance/Quality Control Coordinator.
3. QC Data must demonstrate acceptable assay performance.

6. The name of the testing technologist is linked to each result and control. The testing technologist must initial/sign their worksheets.
7. The director or supervisor or QA/QC technician/designee reviews patient tests results and quality control results of all patient runs and QC analysis on a daily basis. The initials of the director, supervisor, or QA/QC technician/designee on the worksheets document evidence of this review. Review of QC performed in locations other than the laboratory may be performed on a less frequent basis but must be at least once a month.
8. The compiled summary of quality control material is printed (if automated) and reviewed monthly. Evidence of this review is documented by the initials/signature of the director, supervisor, or QA/QC technician/designee on the quality control records.
9. The lot number of reagents and materials used in each assay are recorded directly on the result worksheet or a reagent log, if necessary. This information is retained for a minimum of five years or two years past the closure of the study or indefinitely, determined by the sponsor.

PROCEDURE

A. Quality Control Program

The Quality Control Program can be divided into the following main areas of focus:

1. Internal Quality Control (testing of known materials)
2. Parallel Testing – Validation of new controls and reagent lots
3. Blind or Split Sample Testing
4. Proficiency (External) Testing Programs
5. Quality Control Monitoring – Corrective Action Logs
6. Quality Assurance Program Feedback
7. Quality Control through Preventative Maintenance Program

B. Internal Quality Control (testing of known materials) - Qualitative Test Systems

1. Quality control of assay reproducibility is achieved by testing materials of known reactivity.
2. Qualitative procedures are checked by positive and negative controls.
3. The frequency of controls is dependent on the manufacturer's recommendation as well as the lab confidence/experience with each method. As a minimum requirement, manufacturer recommendations have to be followed. New operators may run controls more frequently than the manufacturer's recommendations. QC must be assayed after maintenance procedures or changes in reagent before patient testing can resume.
4. The number of controls and the frequency of control runs should also be specified in each test procedure.

5. The testing technologist and/or clinic staff is responsible for reviewing and recording the quality control results on the assay worksheet (or equivalent).
6. If the quality control results are within the expected response as stated by the manufacturer and the patient test results appear valid, the testing staff will sign and forward the results to the laboratory supervisor or designee for final review.
7. If the quality control results and patient test results are acceptable, the laboratory supervisor or QA/QC technician/designee will sign and release the test run.
8. All results (quality control and patient) must be reviewed, evaluated and signed by the laboratory supervisor or QA/QC technician/designee before the patient test results can be released.
9. In the event that the lab supervisor or QA/QC technician/designee is unavailable and result release will be delayed, peer review is allowed for release of results. Peer result review must be documented by signature. The laboratory supervisor or QA/QC technician/designee review must be documented as soon as possible.
10. If the quality control results are not as expected as stated by the manufacturer or a potential problem is noted, the testing technologist and/or clinic staff will review the results with the laboratory supervisor or QA/QC technician/designee.
11. All quality control results must be documented including any out-of-range results.
12. Out-of-range results and follow-up action will be documented on the appropriate Corrective Action Log.
13. When a control does not give results as expected or potential problems are noted, the laboratory supervisor or director will make the final decision on the disposition of the run.
14. If the run is considered invalid based on review of the quality control results, all tests must be repeated. The laboratory supervisor or QA/QC technician/designee will review and sign off the corrective action logs once per month. If potential problems exist, the quality control results will be reviewed more frequently.
15. The laboratory supervisor may increase the number of controls, the frequency of controls or request outside testing. There must be sufficient documentary evidence that the laboratory of choice for outside testing can consistently and reliably produce patient results that are not considered to be significantly different. Outside testing laboratories need to be pre-approved by the Network, mentioned in back-up plans and participate in the appropriate proficiency testing. The Laboratory Director or designee is ultimately responsible for making this decision.
16. QC material must be handled exactly as stated in the manufacturer documentation.
17. Any in-clinic testing needs to be reviewed by the appropriate laboratory supervisor or designee on a monthly basis. Any problems noted with the in-clinic testing must be brought to the attention of the laboratory supervisor or director as soon as possible.

C. Internal Quality Control (testing of known materials) - Quantitative Test Systems

1. Quantitative procedures are checked by a low to high range of two to three controls depending on the procedure.
2. The frequency of controls is dependent on the manufacturer's recommendation as well as the lab confidence/experience with each method. As a minimum requirement, manufacturer recommendations have to be followed. All levels of the QC material must be analyzed each time QC is performed. QC must be assayed after maintenance procedures or changes in reagent before patient testing can resume
3. The number of controls and the frequency of control runs should be specified in each test procedure.
4. For commercial quality control material, the manufacturer's ranges can be used until sufficient data has been gathered to enable the establishment of a laboratory or analyzer specific QC range. The mean of the results obtained must lie within the manufacturer recommended ranges unless otherwise stated by the manufacturer. Sufficient data would normally be 20 data points. For hematology systems this may be considered too many, please consult HPTN Central Lab for guidance.
5. The testing technologist is responsible for reviewing and recording the quality control results on appropriate quality control logs. The minimum requirement will include a control log and Levy-Jennings charts. The exception to this requirement is if the analyzer or LIS system keeps track of the quality control. If this is the case, the technologist must sign off on a log sheet indicating the QC was run and within range.
6. If the test system has an automated quality control record function, the control logs and Levy-Jennings charts must be checked each time the controls are run.
7. Patient samples should not be reported before the controls are reviewed and found to be acceptable.
8. If the quality control results are within the established guidelines and no shifts, trends or potential problems are noted on the Levy-Jennings charts, the testing technologist will forward the patient results to the laboratory supervisor or designee for final review.
9. If the quality control results and patient test results are acceptable the laboratory supervisor will sign and release the test run. Generally patient results are considered acceptable if all quality control material fall within the established QC ranges. Lab should have guidance such as a QC Acceptability Policy that describes in detail the criteria for QC acceptability and rejection.
10. All results (quality control and patient) must be reviewed, evaluated and signed by the laboratory supervisor or QA/QC technician/designee before the patient test results can be released.
11. In the event that the lab supervisor or QA/QC technician/designee is unavailable and result release will be delayed, peer review is allowed for release of results. Peer result review must be documented by signature. The laboratory supervisor

or QA/QC technician/designee review must be documented as soon as possible thereafter.

12. If the quality control results are not within the expected ranges and guidelines, the testing technologist will review the results with the laboratory supervisor or QA/QC technician/designee.
13. All quality control result including any out of range results must be documented.
14. Any shifts or trends must be reported to the laboratory supervisor and/or local QA/QC technician. Any shifts or trends must be examined.
15. Out-of-range results and follow-up action will be documented on the appropriate Corrective Action Log.
16. When a control result falls outside the established range or potential problems are noted, the laboratory supervisor or director will make the final decision on the disposition of the run.
    a. Results may be considered acceptable after review.
    b. The review and consideration will be documented on the assay sheet and the corrective action log.
17. If the run is considered invalid based on review of the quality control results, all patient analysis must be repeated
18. The laboratory supervisor or QA/QC technician/designee will review and sign off the quality control data and corrective action logs once per month. If potential problems exist, the quality control results will be reviewed more frequently.
19. The Laboratory supervisor, manager or director may increase the number of controls, the frequency of controls or request outside testing to resolve potential problems.
20. QC material must be handled exactly as stated in the manufacturer documentation.
21. All safety laboratory related QC Data must be submitted on a monthly basis to HPTN Network Laboratory, attention Domestic Quality Assurance/Quality Control Coordinator. (email: centralLab@HPTN.org)
22. QC Data must demonstrate assay performance which is comparable to the performance specifications published by the manufacturer.

D. Parallel Testing – Validation of new controls

Whenever possible, new lots of control material must be assayed in parallel alongside the current in use lot. This is to enable the calculation of laboratory QC ranges and to demonstrate that the QC material is performing as expected. (see HPTN Quality Assessment Policy Version 3 for specifics)

1. Controls for Quantitative Assays:

a. In order to validate new controls, the new lot of controls will be run in parallel with the old lot of controls 2-3 times a day for 5-10 days, to give a minimum of 20 values to enable the calculation of laboratory specific QC ranges. The mean and QC ranges for the new lot of controls will be reviewed and signed off by the laboratory supervisor or director before being put into use.

b. For hematology the new lot of controls should be run in parallel with the old lot of controls to give a minimum of 10 values over a period 5 days if possible. The mean and ranges for the new lot of controls will be reviewed and signed off by the laboratory supervisor or director before being put into use. The use of historic precision data for the calculation of QC ranges is acceptable practice. Please contact the HPTN Network Lab for clarification if needed. (email: CentralLab@HPTN.org)

2. Controls for Qualitative Assays:

Each new lot of QC for qualitative assays must be run and give an expected response. The lot of controls will be reviewed and signed off by the laboratory supervisor or director before being put into use.

E. Internal Quality Control (testing of known materials) - Other Test Systems

Gram Stain

1. Gram stain reagent and procedure will be quality controlled each day of use by including a control slide containing gram positive and negative organisms such as E. coli and Staphylococcus aureus or equivalents.
2. These control slides may be made in-house from known cultures.
3. Acceptance criteria for the gram stain slides will be defined.
4. The slide control results will be documented on a gram stain quality control log.
5. The control log will be initialed and dated by the technologist performing the quality control.
6. The control log will be reviewed and signed at least once per month by the laboratory supervisor or designee.
7. If the control slide stain is not acceptable, check both the staining technique and the stain.
8. Document any problems and corrective action on the gram stain corrective action log.

Differential and/or Malaria Stain

1. The differential stain will be checked each day of staining.
2. The first slide read after staining will be reviewed for correct color formation for the WBC's and RBC's along with excessive background debris.
3. Acceptance criteria for the differential stain will be defined and documented on the control log.
4. The control log will be initialed and dated by the technologist performing the quality control.
5. The control log will be reviewed and signed at least once per month by the laboratory supervisor or designee.
6. If the control slide stain is not acceptable, check both the staining technique and the stain.

7. Document any problems and corrective action on the gram stain corrective action log.

F. Proficiency (External) testing Programs And Blinded/Split Samples

See HPTN Quality Assessment Policy Version 3 for specifics

G. Built in / procedural controls.

Many test systems such as Rapid HIV tests or Pregnancy Tests contain a built in procedural Control Test line. These built in controls indicate whether sample has reached the desired portion of the test strip / reaction chamber. The response obtained from the control portion must as expected for each test strip before patient results can be reported. Please refer to manufacturer kit instructions for specific details.

These built in control lines only indicate that the sample flow has been achieved as desired. Some of the kits do not detect serum or plasma proteins. A positive control line does not indicate that assay has performed as expected with the patient sample.
Quality Control specimens must be assayed for all test systems on a regular basis for all DAIDS related procedures (including FDA Waived test kits).
See HPTN Quality Assessment Policy Version 3 for information

H. Quality Control Monitoring – Corrective Action Logs

1. Corrective Action Logs are maintained for each test and instrument.
2. The corrective action logs are used to document quality control results that fall outside the established ranges, inconsistency in results or problems with test system (reagents, controls, instrument or equipment).
3. The testing technologist is responsible for documenting any problems and corrective action taken on the appropriate corrective action log
4. The logs provide valuable information for troubleshooting test method or instrument problems.
5. The laboratory supervisor or QA/QC technician/designee is to be notified immediately of any problems and will review the corrective action.
6. The corrective action logs will be reviewed and signed off once per month by the laboratory supervisor or QA/QC technician/designee.
7. Corrective action logs should also indicate the status of patient results. ie. whether patient results were reported or not.

I. Quality Assessment

See HPTN Quality Assessment Policy Version 3 for information

J. Preventative Maintenance

See HPTN Quality Assessment Policy Version 3 for information

APPENDICES

1. Quality Control Testing Summary
2. Corrective Action Log

QUALITY CONTROL TESTING SUMMARY

Test	Quality Control		Proficiency Program (check with appropriate DAIDS contractor)		Parallel Testing/New reagent lot Verification	Comments/recommendations
	Material	Frequency	(CAP)	UKNEQAS		
CBC	Low, Normal, High	Daily	X		Overlap Controls	Calibrate per manufactures instructions or every 6 mos
Differential - Manual	Stain Check	Daily	X			
ESR	Low/High	Daily	X			
Malaria Smear – Giemsa/Wright Giemsa	Stain Check	Daily	X			
CD4/CD8	Manufactures Controls	Daily	(x)	X	Reagents	(see IQA Q & A on HPTN website)
Coagulation	Manufacturer controls (normal and abnormal)	Every 8 hours	x		Overlap Controls and Reagents	QC required with every reagent change
Chemistry	Minimum 2 levels	Daily	X		Overlap Controls and Reagents	Calibrate per manufacturers instructions or every 6 mos
HIV-1/2 EIA	Kit controls plus external low level positive control	Run	X		Reagents	Known Neg and positive patients (minimum 1 each)with every new lot
HIV-1/2 Rapid	Commerical or In-House	weekly	X		New kit, new user	New lots need QC'd in lab prior to distribution to clinic. Weekly
HIV-1 Western Blot	Kit: Neg/WeakPos /StrongPos	Run	X			
Urinalysis	Commerical,Normal/Abn	Weekly	X			New lots need QC'd in lab prior to distribution to clinic. Weekly in the lab, clinic per use
Urine Pregnancy	Commerical, Neg/Pos	weekly	X		New lot and new delivery	
RPR	Neg/Weak/Pos	Run	x			

HIV RNA PCR QT	Kit Controls, Neg/L-H Pos	Run	X		Reagents	Recommendation : VQA log controls
GC,Chl PCR QL	Kit Controls, Neg/Pos	Run	X		Reagents	Run controls and 1 pos and 1 neg
Gram Stain	Controls(E.coli and Staph aureus) Stain Check	Daily	x			
Media (cell prep)	Media Check	Per Lot				
Storage-Pla,Ser	Self Audit	Weekly				

CORRECTIVE ACTION LOG

CORRECTIVE ACTION/REMARKS LOG for INSTRUMENT/TEST SYSTEM

_____

Date	Problem/Comments	Initials	Corrective Action/Comments	Initials	Date

Procedure: INSTRUCTIONS FOR HANDLING CAP PROFICIENCY SURVEYS

Prepared by	Date Adopted	Supersedes Procedure #
Sarah Dawson		NEW

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

SITE RESPONSIBILITY

1. Check your site-specific survey schedule on a weekly basis to determine if you should be receiving any surveys for the week. If you have not received a survey within 14 days from the shipping date listed on the calendar, email Debbie Lustig (dlustig@cap.org) (cc your HPTN and/or MTN central laboratory contact and SMILE) to notify her of the missing survey.
2. If you've misplaced your shipping calendar, you can also check the CAP website (www.cap.org) and it will list the most recent surveys shipped to your location. Please include your site's CAP number, the kit, and kit number if available (obtained from the CAP website).
3. Once you submit results to CAP via fax (847) 832-8168 or on-line, check the CAP website to insure all submitted pages have been received. Resubmit any missing pages.

I. Once a proficiency survey arrives:

1. Log the panel in following your specific sops in with the date and time received.
2. Note the acceptability of the specimens.
3. Kit instructions as well as result forms may be downloaded from the CAP website.
4. Run the survey and result it as soon as possible. CAP now includes a date that survey results must be completed and submitted by. The date can be found printed on the result forms as well as on the CAP website.
5. Keep track of who ran the survey (for follow-up purposes as well as documenting competency). The individuals running the survey should be the ones who routinely

perform the testing or who routinely run the analyzers. If possible, the survey should be rotated among staff members trained in the area. It should not be limited to one technologist.

6. The surveys should be run just as if they are patient specimens. No special care should be taken (i.e., running in duplicate or triplicate). Results should not be compared between techs running a survey at your site or at other sites. You should only result surveys performed at your location.
7. When submitting results for your survey, be sure to check that your instrument and method used are the same as on the form.
8. While filling out your on-line result entry, **save** each page as you enter the results. Once all results are saved, you must **approve** "*pending*" pages once you review them for clerical errors. CAP will not grade surveys that remain in "*pending*" status.
9. Once testing on the proficiency samples is complete, store the specimens appropriately. The Hematology specimens should be kept in the refrigerator and Chemistry, Serology, and other non-cellular samples should be stored in the freezer.

II. Once you receive your scores/results from CAP:

1. Using the CAP website will allow you to have faster access to your proficiency results. Scores are posted soon after they are evaluated.
2. Once you log onto the CAP website, click on "e-Lab Solutions."
3. Select your lab and then again click on "e-Lab Solutions."
4. Click on "Evaluations and Reports" (in the center gray box).
5. Choose the survey you wish to view and click on "view details." Under "Available Reports" choose "printed evaluation" and then "view report."
6. Click on PDF Export.
7. Click radio button for "All" and then click on "save report."
8. Click "save" and you will be prompted to name a location to save the file.
9. Click "save." This will allow you to save the file on your computer or on a floppy disc.

CENTRAL LAB/ SITE RESPONSIBILITY

1. SMILE will be overseeing External Proficiency Testing. The HPTN and MTN Central laboraries will be monitoring performance and taking corrective action as needed.
2. SMILE will send a Proficiency Review Form (PRF) for **all analytes** missed on each proficiency survey, even if the overall score is satisfactory.
3. The PRF must be completed by the site.
4. If appropriate, a corrective action must be included and initiated. If an analyte is missed, the above-mentioned retained specimens should be retested and the results documented on the PRF (Proficiency Review Form).
5. Once a site misses the same analyte on consecutive surveys, careful scrutiny by the SMILE is afforded to that panel.
6. If a site misses the same analytes on 3 consecutive panels, the site will not be allowed to perform testing for that analyte for any NIH / DAIDS funded protocol until notified by the Central Lab.
7. The lab's back-up plan must be instituted at that point.
8. If a lab subscribes to proficiency testing from other organizations (i.e., Thistle, AAB, API, etc), SMILE and the Central Lab may consider these results if deemed appropriate.

9. When reviewing your results, pay careful attention to whether or not there is any pattern of results emerging. Specifically look for consistently increased or decreased SDI's (Standard Deviation Indexes).
10. Also look for any trends and shifts in your results that could point to a problem with your instrument.
11. If you have questions or concerns about your instrument or your lab's performance, please contact us at CentralLab@hptn.org and we will provide any assistance that we can.

III. File / Store your results so they are readily available.

1. After reviewing your results (and submitting any necessary PRF's), store or file your results in a format that can be easily accessed by those using the instruments, by PI's and managers, and by lab monitors. CAP provides a notebook yearly that can be used to store results. The information may be helpful in trouble-shooting instrument problems and malfunctions. The results may be stored in electronic format as long as they are readily accessible.

Section 2: Safety

The safety of all staff, patients, and visitors is the most important issue for any laboratory. Laboratories also have a responsibility to make sure that waste from their laboratory does not harm the environment or people in their community. HPTN/MTN has a commitment that all Network laboratories make safety their primary priority.

1. Each laboratory must have written safety procedures and conduct periodic safety training and assessment. The following pages in this section have some example safety SOP's.

2. All laboratories must have a chemical hygiene plan and maintain Material Safety and Data Sheets (MSDS) for all chemicals used in the laboratory. When possible, these should be obtained from the chemical vendor. In cases where this is not possible, they may be obtained from other sources, given that it is for the exact same chemical.

3. All laboratories must follow Universal Precautions and use appropriate personal protective equipment. Further information on this can be found at http://www.cdc.gov/mmwr/preview/mmwrhtml/00000039.htm and in the attached SOP's.

4. Any laboratory which deals with human specimens must have a method for delivering Post-exposure Prophylaxis (PEP) to employees who have had an exposure which may expose them to HIV. Information for how to handle all types of accidents must be communicated to staff and posted prominently in the laboratory.

5. All staff handling specimens must be offered the Hepatitis B vaccine. They may choose to decline the vaccine if they have already had it or for other reasons; this must be documented.

Laboratory Safety	
SOP Number	Total Number of Pages:
Effective Date:	Primary Authors: S.Samiappan, E. Livant
Reviewed by: Dr.P.Balakrishnan	Review Date:
Distributed to:	Distribution Date:

PURPOSE

It is the policy of YRG CARE to provide the employee a safe workplace as protected as possible from exposure to human blood and body fluids and other safety hazards. These policies are designed for the safety of all employees and will be enforced at all times.

SCOPE

This policy applies to all persons performing laboratory procedures or handling specimens for YRG CARE studies in the clinical areas, clinic lab, or Infectious Diseases Laboratory. This document is meant to as a guideline and needs to be complimented by further specific safety training and instruction. It is imperative to be properly trained before using any equipment or performing any procedure. Whenever questions arise, do not hesitate to ask a supervisor for guidance. The safety of one's self and of others is the responsibility of each and every employee.

GENERAL SAFETY REQUIREMENTS

A. Personal
    a. Frequent hand washing is the most important single precaution. Hands should be washed with soap and water after removing gloves, completing a task or after accidental contact with contaminated materials.
B. Dress Code
    a. Shoes must be closed toed and of a fluid resistant material while performing lab work.
    b. Shorts and short skirts may not be worn in the laboratory.
    c. Long hair must be worn up or tied back.
    d. Long beards are not allowed in the laboratory because they pose similar hazards as long hair.

C. Personal Protective Equipment (PPE)
    a. Disposable gloves must be worn whenever handling blood or body fluids.
    b. A long sleeved lab coat or other fluid resistant garment is required whenever handling blood or body fluids.
    c. Face shields and masks may be used when appropriate.
D. Visitors in the laboratory
    a. Outside visitation to the laboratory should be limited to professional reasons to reduce congestion. Do not leave visitors alone in laboratory work areas for extended periods of time.
    b. The use of PPE for visitors is at the discretion of the laboratory staff. This decision should be made based on the proximity that visitor will have to laboratory procedures and risk of contact with infectious agents or chemicals.
    c. Visitors must comply with all other safety regulations outlined in this SOP.
E. Eating, Drinking and cosmetics
    a. It is strictly forbidden to eat, drink, manipulate contact lenses or apply cosmetics in the laboratory.
    b. Food and drink may not be stored in the laboratory.
F. Smoking
    a. Smoking is forbidden in the laboratory.
G. Mouth Pipetting
    a. Mouth pipetting is forbidden in the laboratory.
H. Opening of Test Tubes
    a. Test tubes should be opened away from the technician to reduce exposure. Use shields when available.
I. Physical space
    a. Passageways should be kept clear to avoid accidents and to permit speedy exit in case of emergency.
    b. Candles are not permitted in the laboratory.
    c. Decorations may be posted as long as they do not pose fire or other safety risk.
    d. Lab surfaces (benches, instruments, etc…) should be cleaned frequently with 10% bleach and 70% rectified spirit. This should be generally done at least once per day.
J. Centrifuges
    a. Centrifuges should always be operated closed to prevent aerosol contamination.
    b. Proper maintenance as described by the manufacturer must be followed in order to prevent accidents.
    c. Make sure tubes are balanced to decrease chance of breakage. In case of tube breakage, extreme care should be followed when removing glass or other sharps. Never touch glass with bare hands-use forceps. Disinfect the interior with 10% bleach or other microbicidal agent.
K. Electrical Safety

a. Report any obvious damage or defects to a supervisor. Do not use equipment when in doubt.

Make sure of electrical requirements before changing power source for any equipment. Much of the equipment used in YRG CARE laboratories requires 110 Voltage. Make sure proper voltage regulators, UPS and surge protection is in use.

b. Do not handle electrical equipment while in contact with wet surfaces or floors.

L. Ergonomics
   a. Chronic injury can result from repeated motions. Workspaces should be arranged to facilitate proper motion.
   b. Proper lifting technique must be employed when moving heavy objects.

LAMINAR AIR FLOW HOODS

A. The primary function of this hood is to work in clean environment for specific procedures and to reduce risks from aerosol contamination.
B. The UV light in the air flow hood should be used at night when the lab is unoccupied. Do not stand in front of the hood while the UV light is in use.
C. In case of a power failure, air flow will reverse causing a potentially dangerous situation. If this happens, close the hood door immediately and step away from the hood.
D. Proper training is required before use of the hood.

EXTREME TEMPERATURE HAZARDS

Always get training from experienced personnel before handling extreme temperature devices and materials.

Freezers

A. When handling freezers that operate at very low temperatures (-70°C for example) skin can be burned by prolonged contact with cold surfaces.
B. Take care that exposed skin never comes in contact with these equipments cold surfaces. Gloves and other protective personal equipment should be used to prevent injury.

Liquid nitrogen

A. Keep windows open when transporting liquid nitrogen in a car.
B. Do not touch liquid nitrogen with bare skin. If you get burned by liquid nitrogen, seek medical attention.
C. Safety goggles and gloves must be worn when handling liquid nitrogen.
D. If handling large quantities, a long resistant apron is recommended. Liquid nitrogen can cause severe burns while clothes are being removed.
E. Remove metal jewelry and watches before handling liquid nitrogen.
F. Do not wear pants with cuffs as these can trap liquid nitrogen.
G. Do not use a funnel when transferring liquid nitrogen.

H. Liquid nitrogen can cause explosions when mishandled. Liquid nitrogen must be stored in approved containers following manufacturer's recommendations.
I. See the liquid nitrogen MSDS for more information.

Dry ice

A. Dry ice must be stored in insulated containers.
B. Dry ice must not be stored in airtight containers. As the ice melts, it turns to gas which needs to escape or it will build up pressure.
C. Keep windows open when transporting dry ice in a car.
D. Do not touch dry ice with bare skin. If you get burned by dry ice, seek medical attention.
E. Safety goggles should be worn when handling dry ice.
F. Do not eat dry ice.
G. Be careful to not inhale the vapors from dry ice. If you become short of breath, leave the area of dry ice immediately.
H. Do not place dry ice directly on surfaces. It can cause them to crack.
I. Note: Dry ice is heavier than air and will settle near floors.
J. See the dry ice MSDS for more information.

Heating devices

A. Heating devices include incubators, hot plates and incubators.
B. Heating devices should be positioned in the lab away from flammable articles such as papers or combustible chemicals.
C. Never place items on top of heating devices.
D. If heating devices are of a temperature high enough to cause burns they should be clearly marked to warn other people.
E. Follow manufacturer's recommendations to monitor temperature.
F. Do not use heating devices that may be defective.

Bunsen Burners

A. Place the Bunsen burner away from any overhead shelving, equipment or light fixtures.
B. Remove all papers, notebooks, combustible materials and excess chemicals from the area.
C. Inspect hose for cracks, holes, pinched points, or any other defect and ensure that the hose fits securely on the gas valve and the Bunsen burner.
D. Replace all hoses found to have a defect before using.
E. Use a sparker / lighter with extended nozzle to ignite the Bunsen burner. Never use a match to ignite burner. Have the sparker/lighter ready before turning on the gas.
F. Do not leave open flames unattended and never leave laboratory while burner is on.
G. Shut-off gas when its use is complete.
H. Allow the burner to cool before handling. Ensure that the main gas valve is off before leaving the laboratory.

UNIVERSAL PRECAUTIONS

It is impossible to know the infectious status of the patients supplying all of the biological specimens which are processed by the clinical laboratory. Thus the concept of "Universal Precautions" was developed to reduce the risk of transmission of HIV, Viral Hepatitis and other blood borne pathogens.

Universal precautions apply to blood, other body fluids containing visible blood, semen and vaginal secretions. Universal Precautions also apply to tissues and the following fluids: cerebrospinal, synovial, pleural, peritoneal, pericardial, and amniotic. Universal Precautions do not generally apply to feces, nasal secretions, sweat, tears, urine and vomitus unless they contain visible blood. YRG CARE requires although, that universal precautions will be followed for all specimens taken from human subjects.

All of the above listed specimens will be treated as potentially infectious regardless of the patient's medical condition or recent test results.

Universal precautions state that disposable gloves, personal protective equipment and appropriate barrier protection will be used whenever obtaining specimens from patients and when handling these specimens or items that have been in contact with blood and body fluids.

Under no circumstances should a patient be considered "Noninfectious" or "Safe" and these precautions must never be relaxed.

NEEDLE STICKS AND BLOOD EXPOSURES

An exposure refers to any of the following occurrences:

- A needle stick with a needle that has been in contact with human blood or body fluid
- A cut or other wound where human blood or body fluid was in contact
- A splashing of human blood or body fluid in the face, eyes or mouth
- A large amount of human blood or body fluid coming in contact with intact human skin
- Any incident where there is a risk for transmission of HIV or Hepatitis B or C

In the event of an exposure to blood or body fluid that does not involve injury or skin puncture, immediately scrub the area with soap and water. Rinse and repeat several times. The area should then be cleansed with betadine or other microbicidal agent if available. Let the area air dry after. Report the incident to a supervisor and seek medical attention as warranted.

In the event of a needle stick, cut or injury involving blood or body fluid, immediately squeeze the wound in such a manner as to increase bleeding. (Do not do this if the wound is already bleeding profusely or if the wound is potentially life threatening.) Continue forcing out blood and allow the wound to bleed for 10-15 seconds. Next, scrub the area with soap and water vigorously. Rinse and repeat several times. The area should then be cleansed with betadine or other microbicidal agent if available. Let the area air dry after. Report the incident to a supervisor and seek medical attention.

It may be deemed necessary to provide further medical treatment. In certain cases, the exposed individual may need to be given Post-Exposure Prophylaxis (PEP), presumably within 2 hours of exposure. Thus prompt actions should be taken after these incidents. A list of contact information will be kept in the laboratory message board.

CLEAN UP OF SPILLS

1. Evaluate the spill
    a. Are the materials Innocuous, Corrosive, Flammable, Toxic or Explosive?
    b. Identify all materials by common or chemical name.
    c. Estimate how much is spilled.
    d. Evaluate the degree of danger to staff, students, patients or visitors.
    e. Evaluate the degree of danger to equipment or property.
2. Contain the spill
    a. Utilize any action designed to prevent the spilled material from spreading and causing increased damage.
3. Evacuate
    a. Leave the area if the spill cannot be contained, OR produces irritating odors, OR produces flammable or explosive vapors (extinguish all spark or ignition sources).
4. Clean Up
    a. Spills of innocuous material can be cleaned up by laboratory personnel or housekeeping.
    b. Innocuous spills must be cleaned rapidly to prevent injuries from falls.
    c. Spills of acids, bases and flammables can be cleaned up by laboratory personnel using appropriate neutralizers/absorbents and proper personal protective equipment.
5. Dispose of all contaminated materials properly (see following).

DISPOSAL OF BIOHAZARDOUS WASTE

<u>Solid Waste Disposal</u>

1. Any paper products, cloths, plastic test equipments which have come in contact with blood or body fluids should be considered Biohazardous.
2. All solid bio-waste should be disposed onto a biohazard labeled non-breakable and puncture resistant container.-It should not be filled beyond the 2/3 rd limit.
3. Once in a day or whenever it filled, it should be replaced with new one.
4. The bag must be appropriately sealed to prevent spillage.

5. All sharp must be secured in an approved sharp container prior to disposal.- Finally all solid waste should be disposed by incineration.

Liquid waste Disposal

1. All liquid bio-waste should be discarded on to a leak-proof biohazard labeled container, which should contain ¼ volume of 5 – 10 % hypo-chlorite (Chlorine releasing disinfecting agent).
2. Leave this container for at least 30 minutes to disinfect.
3. Finally discard the liquid bio-waste in to the waste sink and flush the water for at least 5 minutes.

DISPOSAL OF SHARPS

All needles, broken glass or other sharp potentially injurious substances are considered "sharps" and should be discarded in puncture resistant biohazard containers. These should not be overfilled and should be closed when they are ¾ full.

CHEMICALS HYGIENE PLAN

A. General chemical risks:
    a. Burns
    b. Asphyxiation
    c. Poisoning
    d. Chronic exposure.
B. Routes of entry for chemicals to cause injury or disease:
    a. Skin
    b. Eyes
    c. Swallowing
    d. Inhalation
C. All chemicals and reagents must be clearly labeled with date of preparation, expiration date. Some chemical require storage and safety instructions to be clearly listed on the label.
D. Whenever chemicals come in contact with skin, the area should be cleaned thoroughly with soap and water.
E. All chemicals and reagents must be properly stored with respect to heat, light and proximity to other chemicals.

Material Safety and Data Sheets (MSDS)

MSDS must be kept in the laboratory for chemicals used. Any chemical, which is known to be present in the YRG CARE Infectious Diseases Laboratory and the YRG CARE clinic laboratory, must have an MSDS sheet preferably a universal MSDS or from the specific manufacturer or that chemical. When these are not available, an MSDS for the identical chemical from another manufacturer may be substituted. Safety information from package inserts for pre packaged reagents may be used as long as they are considered complete.

A. Responsibilities
    a. All YRG CARE Lab staff that use or come in contact with chemicals must be made aware of the MSDS, their location and purpose.
    b. All YRG CARE Lab staff that use or come in contact with chemicals must do so in a manner compliant with these MSDS so as not endanger themselves or others.
    c. YRG CARE section heads must assist in maintaining a current list of all chemicals used in their respective sections.
    d. YRG CARE Staff shall ensure that labels on containers of hazardous chemicals are not removed or defaced.
    e. YRG CARE laboratory management shall maintain any MSDS for all chemicals and ensure that they are readily accessible during each work shift to laboratory employees when they are in their work areas.

B. The manufacturers of chemicals are responsible to determine the safety issues of these chemicals. YRG CARE will use this information as determined from MSDS to implement safe usage of these chemicals.

C. General information that should be included in MSDS include:

    a. Chemical Name: Means the scientific designation of a chemical in accordance with the nomenclature system developed by the International Union of Pure and Applied Chemistry (IUPAC) or the Chemical Abstracts Service (CAS) rules of nomenclature, or a name which will clearly identify the chemical for the purpose of conducting a hazard evaluation.

    b. Combustible liquid: Means any liquid having a flashpoint at or above 100 deg.F (37.8 deg. C), but below 200 deg. F (93.3 deg. C), except any mixture having components with flashpoints of 200 deg. F (93.3 deg. C), or higher, the total volume of which make up 99 percent or more of the total volume of the mixture.

    c. Common Name: Means any designation or identification such as code name, code number, trade name, brand name or generic name used to identify a chemical other than by its chemical name.

d. Explosive: Means a chemical that causes a sudden, almost instantaneous release of pressure, gas, and heat when subjected to sudden shock, pressure, or high temperature.

e. Exposure Information: Means that an employee is subjected in the course of employment to a chemical that is a physical or health hazard, and includes potential (e.g. accidental or possible) exposure. "Subjected" in terms of health hazards includes any route of entry (e.g. inhalation, ingestion, skin contact or absorption.)

f. Flammables:

   i. Aerosol- Means an aerosol that, when tested by the method described in 16 CFR 1500.45, yields a flame projection exceeding 18 inches at full valve opening, or a flashback (a flame extending back to the valve) at any degree of valve opening.

   ii. Gas- Means, A gas that, at ambient temperature and pressure, forms a flammable mixture with air at a concentration of thirteen (13) percent by volume or less; or A gas that, at ambient temperature and pressure, forms a range of flammable mixtures with air wider than twelve (12) percent by volume, regardless of the lower limit.

   iii. Liquid- Means any liquid having a flashpoint below 100 °. F (37.8 °. C), except any mixture having components with flashpoints of 100 °. F (37.8 °. C) or higher, the total of which make up 99 percent or more of the total volume of the mixture.

   iv. Solid- Means a solid, other than a blasting agent or explosive as defined in 1910.109(a), that is liable to cause fire through friction, absorption of moisture, spontaneous chemical change, or retained heat from manufacturing or processing, or which can be ignited readily and when ignited burns so vigorously and persistently as to create a serious hazard. A chemical shall be considered to be a flammable solid if, when tested by the method described in 16 CFR 1500.44, it ignites and burns with a self-sustained flame at a rate greater than one-tenth of an inch per second along its major axis.

   v. Flashpoint: Means the minimum temperature at which a liquid gives off a vapor in sufficient concentration to ignite.

vi. Hazardous Chemical: Means any chemical which is a physical hazard or a health hazard.

vii. Hazard Warning: Means any words, pictures, symbols, or combination thereof appearing on a label or other appropriate form of warning which convey the specific physical and health hazard(s), including target organ effects, of the chemical(s) in the container(s). (See the definitions for "physical hazard" and "health hazard" to determine the hazards which must be covered.)

viii. Health Hazard: Means a chemical for which there is statistically significant evidence based on at least one study conducted in accordance with established scientific principles that acute or chronic health effects may occur in exposed employees. The term "health hazard" includes chemicals which are carcinogens, toxic or highly toxic agents, reproductive toxins, irritants, corrosives, sensitizers, hepatotoxins, nephrotoxins, neurotoxins, agents which act on the hematopoietic system and agents which damage the lungs, skin, eyes, or mucous membranes. Appendix A provides further definitions and explanations of the scope of health hazards covered by this section, and Appendix B describes the criteria to be used to determine whether or not a chemical is to be considered hazardous for purposes of this standard.

F. See the “Extreme Temperature Hazards” section for additional information on liquid nitrogen and dry ice.

FIRE SAFETY

A. Fire prevention methods
   a. Proper storage, labeling and handling of chemicals
   b. Proper storage of combustibles
   c. Proper maintenance and use of electrical equipment
   d. Proper ventilation
   e. Proper housekeeping
   f. Proper staff education

B. Fire response
   a. Never panic.
   b. In case of fire, it is advisable to leave the area immediately and get help.
   c. Small fires may be put out using fire extinguishers.
      i. All employees should know the location of fire extinguishers.
      ii. Only use fire extinguishers if you have been properly trained.

iii. Never try to extinguish a fire in the laboratory with water because most lab fires are electrical or chemical and water will generally be ineffective and spread the fire.
iv. Fire extinguishers must be properly maintained and charged.
v. Fire extinguishers must be labeled to show dates of next servicing.

d. If you or your clothing catches fire:
   i. Immediately drop to the ground and roll in an effort to extinguish the fire.
   ii. If possible, remove the article of clothing before your skin catches fire.
   iii. Fire blankets may be used if available.
e. It is necessary to familiarize yourself with routes of escape in case of fire. Never use elevators in case of fire.

REPORTING OF ACCIDENTS

Any type of accident or incident which involves breakage of lab equipment, injury, exposure or point towards a potential future risk must be reported. Report these incidents immediately once any necessary medical attention has been given and threat of further injury or damage has been contained.

These reports will be reviewed by a laboratory manager within a week. Periodic reviews should take place to identify preventable risks.

The YRG CARE Infectious Diseases Laboratory Incident report form should be used. (See Appendix A).

REFERENCES

CDC. Universal precautions for prevention of transmission of HIV, hepatitis B virus and other blood borne pathogens in health-care settings. MMWR 1988:37;377-388

NCCLS. Clinical Laboratory Safety; Approved Guideline. NCCLS document GP-17-A (ISBN 1-56238-300-0).

Appendix A: Incident Report Form

YRG CARE
INFECTIOUS DISEASES LABORATORY
INCIDENT REPORT FORM

Date and time of incident: _____
Location of incident: _____
Report completed by/date of report: _____

In case of any incident, please first take appropriate actions to protect the health and well being of yourself and coworkers. Once the situation is under control, report the situation immediately to a supervisor and fill out this form as completely as possible. Use the back of form and additional paper if necessary.

Was anyone injured in incident? Yes ☐ No ☐
If yes, describe injury and action taken: _____

Was there any exposure to blood or body fluids? Yes ☐ No ☐
If yes, describe exposure and action taken: _____

Was any equipment damaged? Yes ☐ No ☐
If yes describe damage and action taken: _____

General description of incident: _____

Reviewed by/Date/Comments: _____

Standard Operating Procedure for Biological Safety Laboratory level 3

Purpose
This standard operating procedure (SOP) describes the procedure for working in a Biological Safety Laboratory level 3 (BSL-3).

Background
BSL-3 materials are high-risk, viable microorganisms associated with human diseases that are serious and potentially lethal. These agents may be hazardous through exposures resulting from autoinoculation, ingestion, or mucous membrane exposure, and particularly infectious through inhalation. *Mycobacterium tuberculosis*, St. Louis encephalitis virus, and *Coxiella burnetii* are examples of BSL-3 agents. At BSL-3 more emphasis is placed on primary and secondary barriers to protect personnel in contiguous areas, the external environment, and the community. This SOP was taken in part from the Biosafety in Microbiological and Biomedical Laboratories 4th edition, 1999.

Biological Safety Laboratory level 3 (BSL-3) procedure:

Safety Equipment
1. Protective disposable laboratory clothing such as solid-front or wrap-around gowns are worn by workers when in the laboratory. Protective clothing is not worn outside the laboratory. Clothing is changed when overtly contaminated.
2. Gloves must be worn when handling infectious materials and when handling contaminated equipment.
3. Frequent changing of gloves accompanied by hand washing is recommended. Disposable gloves are not reused.
4. All manipulations of infectious materials, harvesting of tissues or fluids from infected animals or humans, etc., are conducted in a Class II or Class III biological safety cabinet.
5. When a procedure or process cannot be conducted within a biological safety cabinet, then appropriate combinations of personal protective equipment (e.g., respirators, face shields) and physical containment devices (e.g., centrifuge safety cups or sealed rotors) are used.

Standard Microbiological Practices
1. Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiments are in progress.
2. Persons wash their hands after handling infectious materials, after removing gloves, and when they leave the laboratory.
3. Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the laboratory. Persons who wear contact lenses in laboratories should also wear goggles or a face shield. Food is stored outside the work area in cabinets or refrigerators designated for this purpose only.
4. Mouth pipetting is prohibited; mechanical pipetting devices are used.
5. Policies for the safe handling of sharps are instituted.
6. All procedures are performed carefully to minimize the creation of aerosols.

7. Work surfaces are decontaminated at least once a day and after any spill of viable material.
8. All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method, such as autoclaving. Materials to be decontaminated outside of the immediate laboratory are placed in a durable, leakproof container and closed for transport from the laboratory. Infectious waste from BSL-3 laboratories should be decontaminated before removal for off-site disposal.

Special Practices

1. Laboratory doors are kept closed when experiments are in progress.
2. Laboratory personnel receive the appropriate immunizations or tests for the agents handled or potentially present in the laboratory (e.g., hepatitis B vaccine or TB skin testing), and periodic testing as recommended for the agent being handled.
3. Baseline serum samples are collected as appropriate and stored for all laboratory and other at-risk personnel. Additional serum specimens may be periodically collected, depending on the agents handled or the function of the laboratory.
4. Laboratory personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.
5. Laboratory and support personnel receive appropriate training on the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures. Personnel receive annual updates or additional training as necessary for procedural changes.
6. All personnel should demonstrate proficiency in standard microbiological practices and techniques and in the practices and operations specific to the laboratory facility prior to working in the BSL-3. This might include prior experience in handling human pathogens or cell cultures, or a specific training program provided by the laboratory director or other competent scientist proficient in safe microbiological practices and techniques.
7. A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, pipettes, capillary tubes, and scalpels.
    a. Needles and syringes or other sharp instruments should be restricted in the laboratory for use only when there is no alternative, such as parenteral injection or phlebotomy. Plasticware should be substituted for glassware whenever possible.
    b. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for injection or aspiration of infectious materials. Used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal; rather, they must be carefully placed in conveniently located puncture-resistant containers used for sharps disposal. Non-disposable sharps must be placed in a hard-walled container for transport to a processing area for decontamination, preferably by autoclaving.

    c. Syringes which re-sheathe the needle, needleless systems, and other safe devices are used when appropriate.
    d. Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass should be decontaminated before disposal, and disposed of according to any local, state, or federal regulations.
8. All open manipulations involving infectious materials are conducted in biological safety cabinets or other physical containment devices within the containment module. No work in open vessels is conducted on the open bench. Clean-up is facilitated by using plastic-backed paper toweling on non-perforated work surfaces within biological safety cabinets.
9. Laboratory equipment and work surfaces should be decontaminated routinely with an effective disinfectant, after work with infectious materials is finished, and especially after overt spills, splashes, or other contamination with infectious materials.
    a. Spills of infectious materials are decontaminated, contained and cleaned up by appropriate professional staff, or others properly trained and equipped to work with concentrated infectious material.
    b. Contaminated equipment must be decontaminated before removal from the facility for repair or maintenance or packaging for transport, in accordance with applicable local, state, or federal regulations.
10. Cultures, tissues, specimens of body fluids, or wastes are placed in a container that prevents leakage during collection, handling, processing, storage, transport, or shipping.
11. All potentially contaminated waste materials (e.g., gloves, lab coats, etc.) from laboratories are decontaminated before disposal or reuse.
12. Spills and accidents that result in overt or potential exposures to infectious materials are immediately reported to the laboratory director. Appropriate medical evaluation, surveillance, and treatment are provided and written records are maintained.
13. Animals and plants not related to the work being conducted are not permitted in the laboratory.

Personal Contamination

1. Splashes to face (mucous membranes of eyes, nose and mouth)
    a. Use the eyewash for 15 minutes to flush exposed area.
2. Hands or other exposed skin
    a. Wash with antiseptic or soap and cool water.
3. Needlesticks and Puncture Wounds
    a. Wash with antiseptic or soap and cool water.
4. During normal business hours, report your injury to your supervisor then go to the Health Clinic at Magee-Womens Hospital, rm 4515 ext. 4445, and report to Gina Guldenschuh or nurse in charge to receive appropriate medical treatment.

5. During off hours, go to the Magee-Womens Hospital Emergency Room to receive appropriate medical treatment. Report your injury to your supervisor and Gina Guldenschuh at the next available opportunity.

NEW EMPLOYEE HEALTH AND SAFETY CHECKLIST

The completion of this checklist will ensure that you have been properly advised and trained regarding safety-related issues in your laboratory area. The signed and dated checklist will be retained by your supervisor as part of your personnel file.

_____ Scheduled for baseline serum sample.
_____ Scheduled Hepatitis B vaccination.
- _____ Received first dose
- _____ Received second dose
- _____ Received third dose

_____ Discussed use of universal precautions when working with human blood and body fluids.
_____ Discussed infectious agents specific to the work area (HIV-1, HIV-2, HTLV-I/II).
_____ Discussed required use of personal protective equipment (lab coats, gloves, eye wear, respirators, chemical fume hood, biosafety cabinet use, eyewash stations, showers).
_____ Provide appropriate personal protective equipment (lab coats, eye goggles, masks, respirators).
_____ Discussed appropriate laboratory attire.
_____ Discussed radioisotopes and obtained a radiation exposure monitoring badge.
Biosafety/Radiation Training: _____ Scheduled _____ Completed
_____ Discussed procedures for waste disposal:
- _____ Infectious agents
- _____ Non-infectious agents
- _____ Sharps
- _____ Hazardous chemicals and ultra-violet light exposure

_____ Discussed hazardous chemical inventory and discussed safe use of hazardous chemicals in the Laboratory.
_____ Discussed laboratory-specific labeling of chemicals and infectious agents.
_____ Reviewed laboratory procedures for transfer and storage of hazardous chemicals.
_____ Reviewed emergency response procedures:
- _____ Infectious material spill, release, or exposure
- _____ Hazardous chemical spill or release
- _____ Fire or explosion
- _____ Medical emergency

_____ _____
Laboratory Worker Date Supervisor Date

Section 3: Specimen Management

Quality specimens are essential for any laboratory. Key elements of specimen management include collection, transport, storage and shipping. Also essential for clinical trials is "Chain of Custody" (COC) which refers to the tracking of specimens and results.

Specimen collection must be performed only by properly trained personnel. Most urine and blood specimens can be collected by laboratory staff or nurses. Some specimens such as vaginal swabs must be collected by nurses or physicians. Highly invasive procedures such as lumbar puncture must be performed by a physician. It is essential that staff are aware of proper collection techniques, container types, special requirements and proper care for the patient. The specimens must be transported within predefined time limits to the laboratory under proper conditions.

All HPTN and MTN laboratories will use the Laboratory Data Management System (LDMS) for specimen storage, shipping, and in some cases, assays. Frontier Science Foundation (FSTRF; http://www.fstrf.org/ldms/) is the technical support service for LDMS. Laboratories must have adequate refrigerator and freezer space to store all the specimens necessary. These pieces of equipments must have their temperature monitored 7 days a week, 365 days a year.

Each laboratory must have at least 2 staff that has passed International Airline Transport Association (IATA) training. All international shipments must meet IATA requirements as well as any local requirements. Domestic shipments must follow local regulations.

All HPTN/MTN sites must have a study specific SOP for Chain of Custody in place before study activation. COC must track when specimens are transferred between clinics, processing units, and laboratories and when results are released to the clinic. Internal movements (within the same laboratory) of specimens and results do not need to be tracked. Laboratories with Laboratory Information Management Systems may be able to track this information electronically. Other laboratories will need to devise a system of log books. These systems must capture the personnel receiving/delivering specimens or results and the time and date of the transfer.

Instituto de Pesquisa Clinica Evandro Chagas - FIOCRUZ

PROCEDURE: CHAIN OF CUSTODY

HIV/AIDS Clinical Trials Unit
HPTN 10-001
Standard Operating Procedure
Number 5.1
Version 1.0
Revision History:

Created by:	Reviewed by:	Approved by:
Date: Signature:	Date: / / Signature:	Date: / / Signature:

Revision History:	**Version**	**Effective Date**	**Description**
	1.0	20/Aug/05	First Approval
Next Review:	**Up to 1 year after the approval date**		

Scope:
This SOP applies to all staff members involved in the collection, transport or processing of HPTN 052 specimens and the release of results.

Purpose:
It is necessary to document the transfer of specimens and the release of results to reduce the loss of samples and ensure that results are accurate.

Policy:
All specimens are collected, processed, stored, and shipped in accordance with protocol specifications. All clinical and laboratory personnel must follow the procedures described in this document. All clinical and laboratory personnel are trained in the proper and safe way to handle infectious specimens in compliance with federal and protocol regulations and guidelines.

Procedures:

1. Specimen Collection
    a. Most specimens are collected at the Fiocruz clinic. Viral load, CD4 and HIV ELISA and Western Blot tests will also be collected at HSE and HGNI.
    b. Tests will be ordered by clinic staff.

c. All specimens are collected per current specimen collection procedures.
d. Urine pregnancy and vaginal wet mount testing is done at bedside and does not require transfer of specimens between staff. No chain of custody documentation is required for these specimens.
e. All other specimens must be sent to different laboratories for testing and/or processing and storage.

2. Specimen dispatch from the clinics
    a. Specimen dispatch and results receipt registers will be maintained for each specimen processing and testing location. In some cases, more than one register will be maintained for a location.
    b. After collection, each specimen will be logged into the appropriate register by the clinic staff.
    c. When the specimens are dispatched to the laboratories, this will be recorded in the appropriate register.

3. Specimen Processing and Testing Locations Registers Summary
    a. Laboratory of AIDS and Molecular Immunology (IOC)
        i. Register A
            1. HIV RNA Viral Load Testing
            2. CD4+ Lymphocytes analysis
            3. HIV Genotyping
        ii. Register B
            1. LDMS storage
            2. PBMC processing

    b. Instituto de Pesquisa Clinica Evandro Chagas (IPEC)
        i. Register A
            1. Hematology
            2. Chemistry
            3. Urinalysis
        ii. Register B
            1. Parisitology
            2. Bacteriology
            3. HIV testing (ELISA and Western Blot)
        iii. Register C
            1. Syphilis testing (RPR and TPHA)
            2. Hepatitis B testing

    c. Diagnósticos da América
        i. Register A
            1. CT/NG analysis by nucleic acid amplification
            2. Carbon Dioxide

4. Specimen Receipt at IOC
    a. All specimens will be received in the laboratory.
    b. There are specimen receipt and results dispatch registers which correspond to those in the clinic.
    c. When Specimens are received, this information will be recorded in the appropriate register by the lab staff.

5. Specimen Receipt at IPEC
    a. Specimens are first received in the central specimen collection area.
    b. There are specimen receipt and results dispatch registers which correspond to those in the clinic.
    c. When specimens are received, this information will be recorded in the appropriate register by the specimen collection staff.
    d. Registers will be maintained in each department to record the receipt of the specimen, which will be logged in by the departmental lab staff, and the release of results to the specimen processing area or directly to the clinic.
6. Specimen Receipt at Diagnósticos
    a. When specimens are received at Diagnósticos, the information will be recorded per Diagnósticos procedures.
7. Results release from IOC
    a. Results are delivered directly from the laboratory to the clinic.
    b. When the results are released from the laboratory, this is recorded in the appropriate register where the specimen was logged when it arrived.
    c. When the results are received at the clinic, this is recorded in the appropriate register where the specimen was logged when it was sent from the clinic.
8. Results release from IPEC
    a. Results will first be released from the laboratory to the specimen processing area. They will then be sent to the clinic.
    b. All departments except immunology will send their results to the specimen receiving area first. When the results are released from the laboratory, this is recorded in the appropriate register where the specimen was logged when it arrived.
    c. When the results are received at the processing area, this is recorded in the appropriate register where the specimen was logged when it was sent from the clinic.
    d. Immunology will send its results directly back to the clinic. This information will be recorded in the immunology log book.
    e. When the results are released from the processing area, this is recorded in the appropriate register where the specimen was logged when it arrived.
    f. When the results are received at the clinic, this is recorded in the appropriate register where the specimen was logged when it was sent from the clinic.

9. Results release from Diagnósticos

a. Diagnósticos will maintain their own internal documentation of release of results.
b. When the results are received at the clinic, this is recorded in the appropriate register where the specimen was logged when it was sent from the clinic.

SAMPLE COLLECTION AND TRANSPORT		
Title: Sample Collection and Transport	SOP Number:	Total Number of Pages:
Effective Date:	Supersedes:	Primary Author: S.Hanas
Revision History: Jan, Mar, Jul, Oct 2004		
Reviewed by: Dr. P. Balakrishnan, E. Livant	Review Date:	
Approval Signature and Date:		
Distributed to: All HPTN 052 Lab & Admn. members	Distribution Date:	

SCOPE

This SOP applies to all collection and transport of human specimens for testing from patients and study participants at YRG CARE. It must be followed by all staff involved in clinical sample collection and transportation.

PURPOSE

It is vital that high quality specimens of the correct type are delivered to laboratory in a prompt fashion in order to generate accurate results. To prevent pre-analytical errors in the procedures for collection of high quality samples, all technical staff involved in clinical sample (blood, endocervical swab, urine, pus, sputum, pleural fluid, CSF, etc.,) collection and transport need to adhere to standard procedures. *See appendix A for a summary of Specimen types and associated tests.*

SPECIMEN LABELING

One of the most frequent and potentially most serious sources of laboratory error is mislabeled specimens. It is rarely possible for a technician to determine a specimen was incorrectly labeled from the results generated. In certain cases, physicians may detect errors based on previous results or the patient's physical condition. Thus some errors may occur without detection. Results from mislabeled specimens can cause improper treatment of patients and adverse health events and even death. Misreporting of certain illness such as HIV and other STI can have serious psychological and social adverse

consequences. Thus proper labeling is the number one priority of all staff that collects clinical specimens.

1. Always positively identify a patient before collecting a specimen.
    a. Always check a physical piece of identification when available.
    b. It is advisable to ask the person's identification before each specimen collection even if you know the individual.
    c. When a person does not have identification ask "What is your name?" instead of asking "Is your name…?"
2. Specimens may only be labeled immediately collection or immediately after.
3. Never leave specimens unlabelled after collection. They can be confused with other specimens.
4. YRG CARE patient sample labels must include patient Identification number and accession number.
5. For HPTN and ACTG specimens, labels must include a Patient Identification Number (PID) and date.
6. For other studies, specific labeling requirements may apply.
7. If labels are handwritten they must be legible.
8. A register is kept in the clinic laboratory where specimens are collected to identify which staff member has collected each specimen.

SAFETY

1. All specimens collected from patients must be considered potentially infectious.
2. Universal precautions must be followed for all specimen collection procedures.
3. The use of gloves and frequent hand washing is the best method to prevent disease transmission for both patients and staff.
4. Puncture resistant sharps boxes must be used for any procedure involving needles or sharp objects.
5. Refer to the current YRG CARE Safety SOP for more detailed information.

SPECIMEN COLLECTION PROCEDURES

A. VENOUS BLOOD COLLECTION

Materials

1. Vacutainer holders
2. Tourniquets
3. Syringes
4. Appropriate vacuum blood tubes
    a. Purple Top: EDTA
    b. Grey tops: Sodium fluoride
    c. Red Tops: No additive
    d. Blood culture tubes
5. Gauze
6. Sterile gauze for blood cultures
7. Needles
8. Surgical spirits
9. Disposable gloves
10. Puncture resistant needle boxes
11. Johnson/micropore adhesive tape

Before Drawing Blood

1. Proper training and practice is necessary before staff can be allowed to perform phlebotomy procedures unsupervised.
2. In cases of failed phlebotomy, do not re-attempt phlebotomy more than three times. It is recommended to consult a second staff member in difficult cases. -It is crucial to label the samples correctly.
3. Before collection, always wash your hand and wear gloves.
4. Check the lab requests carefully and choose the appropriate tubes. The general draw order is red, grey and purple.
5. The patient must be properly positioned. Never attempt to draw blood from a person while they are standing. They must be seated or prone.
6. Arrange all materials so that they are easily accessible and the phlebotomy can be performed without undo risk to the technician or patient.

Venipuncture Procedure-Routine

1. Place a tourniquet around the arm above the elbow.
2. Request the subject to vigorously open and close the hand several times.
3. Request the subject to close the fist with thumb inside.
4. Locate a prominent vein.
5. Cleanse the skin over the vein at the elbow with (antiseptic) surgical spirit.
6. Allow the spirit to dry or wipe with clean gauze.
7. Draw the skin tense over the vein with the thumb of the left hand. Do not touch the point of venipuncture.
8. With a quick thrust, puncture the skin and the vein with the level of the needle facing up.
9. Do not move the needle or needle holder when the needle is in the vein.
10. For serum, the RED top Vacutainer containing no additives tube is inserted into the specific needle device. When it is in position, blood would flow into the tube. When the level marked in the tube is reached, the tube should be removed.
11. For plasma or whole blood, insert the PURPLE top Vacutainer containing EDTA tube is inserted into the specific needle device. When it is in position, blood would flow into the tube. When the level marked in the tube is reached, tube should be removed and invert the tube gently no more than five times to mix blood and additive.
12. For blood sugar test, GREY top Vacutainer containing Sodium Fluoride tubes are used, collection and mixing is done as mentioned above.
13. When desired blood volume has been drawn, Release the tourniquet and ask the participant to release the fist.-Remove needle, then press sterile cotton on puncture for five minutes
14. Apply a piece of Johnson/micropore adhesive tape.
15. Transfer tubes for serum separation on to a slant rack and leave them undisturbed for at least 30 minutes before separation.
16. The plasma tubes are kept straight in the racks for about 30 minutes before separation.

17. Whole blood tubes are transported as soon as possible after mixing.

Special Venipuncture Procedure-Lactate

1. Stringent sample preparation and handling techniques are necessary to prevent changes in lactate concentrations, both during and after the blood is drawn.
2. Participants should be fasting and at complete rest for at least 2 hours to allow lactate concentrations to reach steady state.
3. Venous specimens must be obtained without the use of a tourniquet or immediately after the tourniquet has been applied. Alternatively, the tourniquet should be removed after the puncture has been performed, and the blood should be allowed to circulate for few minutes before the sample is withdrawn.
4. Draw blood with a minimum of stasis from fasting, resting participants into tubes containing Sodium fluoride as anticoagulant. Mix well by gentle inversion 10-15 times. Cool blood tube in an ice bath.
5. Within 30 minutes separate the plasma from blood by centrifugation at 400x g for 10 minutes. Avoid excessive forces, which contribute to haemolysis.

Special Venipuncture Procedure-Blood for Bacterial Culture and Sensitivity

1. Whole blood may be required for bacteriological examination. The following factors may be considered by the physician when ordering the test:
    a. Collect blood during the early stages of disease since the number of bacteria in blood is higher in the acute and early stages of disease.
    b. Collect blood during paroxysm of fever since the number of bacteria is higher at high temperatures in participants with fever.
    c. In the absence of antibiotic administration, 99% culture positivity can be seen with three blood cultures.
2. The venipuncture area must be vigorously cleaned to avoid contamination of the sample with skin flora.
3. Always draw the blood culture sample first. Other specimens may be drawn next in their normal order. 2 blood culture vials are taken for each draw.
4. Venipuncture Procedure

a. Clean the tops of the blood culture bottles where the blood will be injected. Do not touch this area again. Position the bottles so that the blood may be transferred easily with minimal risk.
b. This procedure must be performed using a syringe.
c. Apply tourniquet and locate vein.
d. Scrub a larger than normal area using surgical spirit and sterile gauze. It is recommended to use a second antiseptic such as betadine.
e. Do not touch the skin near the vein.
f. Insert the needle into the vein and perform phlebotomy.
g. Take care to transfer blood without touching needle to any other surface.

5. Small children usually have higher number of bacteria in their blood as compared to adults and hence less quantity of blood needs to be collected from them.

Table 1. Volume of blood to be collected at different ages:

Age	**Volume in 2 bottles**
< 2 years	2 ml
2-5 years	8 ml
6-10 years	12 ml
10 years	20 ml

B. **BLOOD SMEAR FOR MALARIAL PARASITES**

1. Materials
   a. Alcohol to clean finger.
   b. Cleaned microscope slides
   c. Fingerstick device
   d. Gauze
   e. Johnson/micropore adhesive tape
   f. Disposable gloves
2. Timing of sample collection:

a. Whenever possible, specimens should be collected before treatment is initiated. In suspected malaria and babesiosis cases, blood smears should be obtained and examined without delay.
b. Since the parasitemia may fluctuate in many, multiple smears might be needed. These can be taken at 8 to 12 hour intervals for 2 to 3 days.

3. Finger stick samples are preferred because there is higher parasite density in surface capillary blood samples than in from standard venous samples.
4. Thick smears: Thick smears consist of a thick layer of dehemoglobinized (lysed) red blood cells (RBC's). The blood elements (including parasites, if any) are more concentrated (app. 30X) than in an equal area of a thin smear. Thus, thick smears allow a more efficient detection of parasites (increased sensitivity). However, they do not permit an optimal review of parasite morphology. For example, they are often not adequate for species identification of malaria parasites: if the thick smear is positive for malaria, the thin smear should be used for species identification. At least 2 smears per participant should be prepared.
5. Thin smears: Thin smears consist of blood spread in a layer whose thickness decreases progressively toward the feathered edge. In the feathered edge, the RBC's should be in a monolayer, or even more distant from one another. At least 2 smears per participant should be prepared. Thin smears are the same as the type used in hematology for manual White Blood Cell Differentials.
6. Fingerstick Procedure:
    a. Label pre-cleaned slides, pre-labeled with the participant's ID, date and time of collection.
    b. Choose a location on the side of the finger near the tip.
    c. Clean the site well with alcohol; allow to air dry.
    d. Prick the side of the pulp of the 3rd or 4th finger.
    e. Wipe away the first drop of blood with clean gauze.
    f. Prepare at least 2 thick smears and 2 thin smears (see below).
    g. Bandage the pricked finger with gauze and tape.
7. Thick smears:
    a. Place a small drop of blood in the center of the pre-cleaned, labeled slide.

b. Using the corner of another slide or an applicator stick spread the drop in a circular pattern until it is the size of a 25 paise coin (1.5 cm).
c. A thick smear of proper density is one, which, if placed (wet) over newsprint, allows you to barely read the words.
d. Lay the slides flat and allow the smears to dry thoroughly. (Protect from dust and insects) Insufficiently dried smears (and/or smears that are too thick) can detach from the slides during staining. The risk is increased in smears made with anticoagulated blood. At room temperature, drying can take several hours; 30 minutes is the strict minimum; in that latter case, handle the smear very delicately during staining.
e. Be careful that ants to not come in contact with the slides as they may be attracted to blood.

8. Thin Slides
    a. Place a small drop of blood on the pre-cleaned, labeled slide
    b. Bring another slide at a 30-45° angle up to the drop, allowing the drop to spread along the contact line of the 2 slides.
    c. Quickly push the upper (spreader) slide toward the unfrosted end of the lower slide.
    d. Make sure that the smears have a good-feathered edge. This is achieved by using the correct amount of blood and spreading technique.
    e. Allow the thin smears to dry (at least 5 minutes).
    f. Fix the smears by dipping them in absolute methanol.
9. The smears will be sent to Technicians in the Main Lab and he will record and send the specimens to the Microbiology section for the staining and microscopic examination.

C. **URINE**

1. Materials
    a. Clean non-sterile disposable screw top urine cups for urinalysis and pregnancy testing

   b. Sterile disposable urine cups screw top for culture and Chlamydia and Gonorrhea Nucleic Acid Amplification (CT/NG NAA).

2. Urine Collection for urinalysis or pregnancy testing
   a. It is advisable to use first morning specimen because it will have the highest concentration of hCG and other analytes. When this is not possible, attempt to obtain urine at least 2 hours after the last void. If these are not available accept any random specimen.
   b. Instruct the patient to enter the bathroom and urinate into the cup until it is ¾ full.
   c. Collect the specimen from the patient. Make sure the lid is firmly secured. Do not carry by the lid.
3. Urine Collection CT/NG NAA (Men only)
   a. It is advisable to use first morning specimen because it will have the highest concentration of microorganisms. When this is not possible, attempt to obtain urine at least 2 hours after the last void. If these are not available accept any random specimen.
   b. Instruct the patient to enter the bathroom and urinate into the cup without cleaning his penis.
   c. The patient should be instructed to only fill about 30 mL in the cup. Show the patient how much should be put in the cup.
   d. Collect the specimen from the patient. Make sure the lid is firmly secured. Do not carry by the lid.
4. Urine Collection for Culture
   a. It is advisable to use first morning specimen because it will have the highest concentration of microorganisms. When this is not possible, attempt to obtain urine at least 2 hours after the last void. If these are not available accept any random specimen.
   b. Instruct the patient to enter the bathroom and clean his penis or her labia.
   c. The patient should urinate for a few seconds into the toilet, stop and then urinate into the cup.

d. Collect the specimen from the patient. Make sure the lid is firmly secured. Do not carry by the lid.

D. **SWAB COLLECTION**

Vaginal Swab for Wet Mount for TV, Candida and BV

1. Materials
    a. Saline
    b. Slides
    c. Coverslips
    d. 2 Dacron swabs
    e. Transport tubes pre-filled with 5 drops of saline.
    f. Warm water for lubrication
    g. Speculum
    h. Disposable gloves
2. Arrange equipment.
3. Arrange the patient on the table or chair with legs in the stirrups.
4. If there is a visible discharge, the doctor can take the specimen without insertion of speculum.
5. (If needed) Lubricate the speculum with warm water. This will allow easier insertion and reduce discomfort for the woman from cold.
6. Insert the speculum.
7. The specimen can be taken from the posterior blade of the speculum or from the vaginal wall if the patient has recently douched. Take two swabs.
8. Swab the first slide with the swab. Apply one drop of 10% KOH to the first slide and smell for a fishy odor. Record this result on the appropriate form. Apply a cover slip.
9. Swab a second slide with the same swab. Store it in the a transport tube.

Genital Ulcer swab for Multiplex PCR

1. Materials

   a. Dacron swabs from the Amplicor Chlamydia Collection Kit (Roche Molecular Systems, Branchburg, NJ, U.S.A.)
   b. Tubes containing 1 ml of the sample transport medium (STM)
   c. Disposable gloves
   d. Speculum
   e. Warm water
   f. Collection tray
2. This sample is taken when a genital ulcer is visualized. Do not take this specimen from a genital laceration or abrasion.
3. Position patient on examination table in the lithotomic position.
4. Arrange equipment.
5. The speculum will be use only if the ulcer is on the interior of the vagina.
6. (If needed) Lubricate the speculum with warm water. This will allow easier insertion and reduce discomfort for the woman from cold.
7. If the ulcer is clean and free of pus, the doctor will swab it directly.
8. If there is pus, the doctor will first remove the pus, and then swab the ulcer.
9. If the ulcer is crusty, the doctor will first remove the crust, and then swab the ulcer.
10. The swabs are placed into a tube and the tops of the swabs broken off. The tubes are then capped and labeled.

<u>Endocervical Swab for CT/NG NAA</u>

1. Materials
   a. Dacron swabs
   b. Disposable gloves
   c. Speculum
   d. Warm water
   e. Collection tray
2. Position patient on examination table in the lithotomy position.
3. Arrange equipment.

4. (If needed) Lubricate the speculum with warm water. This will allow easier insertion and reduce discomfort for the woman from cold.
5. Insert speculum. Make visible the cervix.
6. Clean mucous secretions from the cervix if necessary.
7. Take the swab from endocervical discharge or cervix.

Skin lesions for Fungal wet mount

1. Materials
    a. Dacron Swabs
    b. Disposable gloves
2. Open the vesicle and absorb exudates onto a collection swab.

E. **SEMEN**

1. Semen collection is only performed for certain study protocols.
2. Materials
    a. Clean non-sterile disposable screw top urine cups.
    b. Antiseptic towelettes.
    c. Disposable gloves.
3. Prior to semen collection, all semen collection containers should be labeled.
4. Provide participant with a labeled semen collection container.
5. Instruct participant to wash his hands and penis and then use an antiseptic towelette to wipe the head of the penis including the opening. If the participant is uncircumcised, ask him to pull back the foreskin before cleaning the head and opening.
6. Provide the participant with a private location and instruct him to masturbate, collecting the entire specimen in the container. If the specimen is collected at home, the specimen should be returned to the clinic within 2 hours.
7. Collect the specimen from the participant.
8. Sample will be processed and sent for storage to main lab.

F. **CERVICAL SECRETIONS**

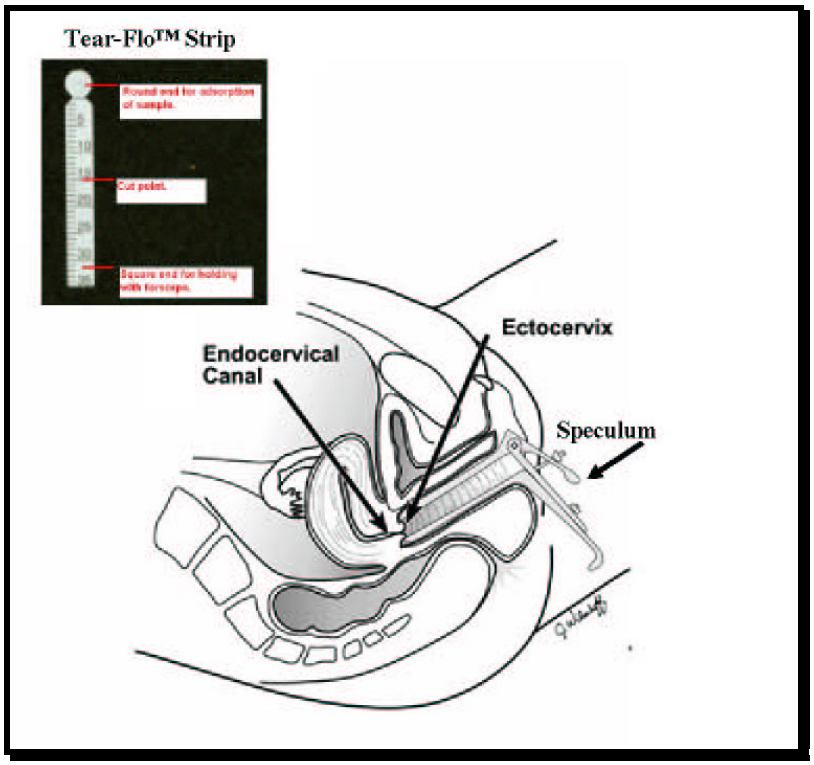
1. Cervical secretions are collected to quantitate cervical HIV-1 RNA

2. Materials
    a. Tear-Flo™ strips
    b. Speculum
    c. Disposable gloves
    d. Scissors
    e. Cryovials
    f. Examination table
    g. 1X Nucleic Acid sequence Based Amplification (NABSA) buffer
        i. Must be free from crystals before use. Keep at room temperature until crystals disappear. If crystals persist, vortex away crystals.
        ii. Store refrigerated at 2°C-8°C until expiration date.
3. The participant must refrain from any kind of sexual activity, douching, and inserting any intravaginal products for at least 48 hours prior to the collection of cervical specimens.
4. Do not collect specimens while the patient is menstruating.
5. Tear-Flo™ will be used as wicks to collect primarily cell-free virions from the endocervical canal fluid in the following manner.
6. Help the participant assume the lithotomy position.
7. Gently insert an unlubricated and appropriately-sized speculum into the vagina and lock it into place.
8. Use a large cotton-tipped swab to gently remove excessive mucus in the vagina and the cervical os before inserting the Tear-Flo ™.
9. Using forceps, gently insert two aligned Tear-Flo ™ simultaneously into the vagina, place through the cervical os into the distal endocervical canal, and hold in place to absorb sample. Maintain the Tear-Flo ™ inside the endocervical canal until the absorbed secretions reach past.
10. Each Tear-Flo ™ will absorb approximately 12 µL of specimen. Absorption usually takes approximately one minute, but may take a little longer.
11. Hold the round end of the two strips over and slightly inside one labeled (participant ID label and hand-written date) plastic transport tube (2.0 mL cryovial) containing 500 µL of NASBA buffer. Cut the strips at the “15” mark

with scissors, allowing the round ends to fall into the cryovial. Cap and invert the sample, or vortex it for 5 seconds

12. Two sets of specimens (2 Tear-Flo ™ each) should be collected: one for specimen storage and the other for cervical HIV viral load.
13. Tear-Flo ™ in NASBA buffer are stable for 48 hours at 15°C -30°C and for 14 days at 2°C -8°C, so keep the sample refrigerated or on ice until it is frozen at -80°C.

Figure 1: Tear-Flo ™ Strip Use



G. **CEREBROSPINAL FLUID (CSF)**

Pre collection considerations

1. CSF is collected through lumbar puncture. It is one of the most invasive specimen collection techniques and must only be performed by a trained physician.
2. CSF specimens must be handled with extreme care. CSF is usually collected when a patient exhibits symptoms suggestive of serious illness such as

Meningitis. The collection of CSF requires discomfort and potential side effects for the patient. These include:

a. Headache
b. Pain during the test
c. Bleeding into the spine canal
d. Hypersensitivity reaction to the anesthetic
e. Damage to the spinal cord
f. Brain herniation

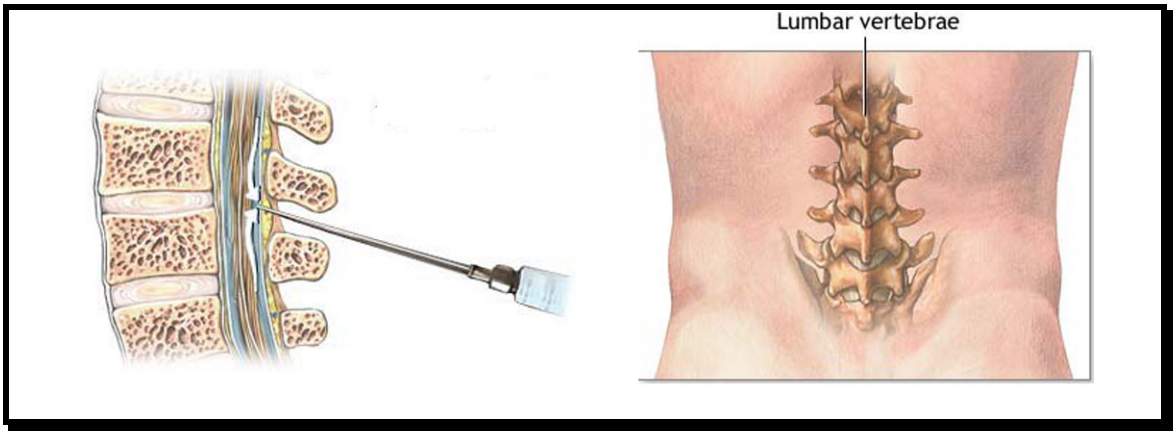
3. Materials
    a. Equipment tray
    b. CSF collection tubes
    c. Anesthetic
    d. Syringes or CSF collection kit
    e. Needles
    f. Disposable gloves
    g. Skin disinfectant
    h. Gauze
    i. Bandages
4. The tubes need to be labeled in the order they are collected. The lab will decide which tubes will be used for which tests based on how many tubes are sent.
5. The lab should be notified before the procedure is started so they will be ready when the specimens arrive.

Collection procedure (Adult)

1. Have the patient lay on their side and curl into the fetal position
2. Locate the area to be pierced, usually between the third and fourth vertebrae.
3. Disinfect the area.
4. Insert the needle into the subarachnoid space. Transfer CSF into the tubes. Collect only the amount necessary.
5. Clean the wound and apply a bandage.

6. Have the patient remain laying down for at least 30 minutes. The patient should remain under observation after the procedure for several hours.

Figure 2: CSF collection



H. SPUTUM FOR AFB CULTURE AND FUNGAL CULTURE

1. Materials
    a. Disposable gloves
    b. Disposable, wide mouth sterile sputum container
2. Give the participant a sputum container with the laboratory serial number written on it. Show the participant how to open and close the container and explain the importance of not rubbing off the number written on the side of the container.
3. Instruct the participant to inhale deeply 2-3 times, cough up deeply from the chest, and spit in the sputum container by bringing it closer to the mouth.
4. Make sure the sputum sample is of good quality. A good sputum sample is thick, purulent, and sufficient in amount (2-3 ml).

I. **STOOL**

1. Materials

a. Dry, large, leak proof container uncontaminated by urine, water, soil or other extraneous material.
b. Pre-labeled specimen container with formalin.
c. Disposable gloves.
d. Plastic spoons
e. Disposable gloves

2. Provide the patient with a pre-labeled container.
3. Instruct the patient to defecate into the large container. The patient should attempt to not get any urine into the container.
4. Instruct the patient to transfer the stool into the pre-labeled specimen container.
5. Instruct the patient to tightly close the stool container and wash his/her hands with soap and water. Collect the container from patient.

SPECIMEN TRANSPORT PROCEDURES

Specimen Transport Containers

1. Specimens must not be transported from the clinic to laboratories by hand.
2. Specimens must be placed in a biohazard bag or a hard cooler (recommended).
3. Specimen transport containers must be clearly marked with biohazard labels.

Specimen Transport Conditions

1. Specimens should not be subjected to excess shaking or vibrations.
2. Certain Specimens need to be transported at a specific temperature. (See Table 2)
3. Specimens must be processed or transported to the laboratory where they will be processed within the time listed in Table 2.
4. Whenever it is possible to deliver specimens faster than indicated in Table 2, do so.
5. Notify the technician of any problems that occurred in the collection or transportation of specimens.
6. Specimens listed as “immediate” have high priority and short viability and should be transported without delay.
7. STAT specimens must be transported immediately.

Table 2: Specimen Transport Conditions

Specimen	Transport Temperature	Maximum Transport Time
EDTA Whole Blood	Room Temp[1]	1 Hour
Serum or Plasma (Routine)	Room Temp	Must be centrifuged within 1 hour.
Plasma (Lactate)	Refrigerated[2]	Immediate
Finger Stick Slides	Room Temp	2 hours
Urine (Culture or CT/NG NAA)	Refrigerated	1 day
Urine (Other)	Room Temp	1 Hour
CSF	Room Temp	Immediate
Swabs (Wet Mount)	Room Temp	Immediate
Swabs (Other)	Room Temp	1 Hour
Sputum	Refrigerated	1 Day
Stool	Room Temperature	1 Day
Semen	Room Temperature	Immediate
1. 15° C -30° C: If ambient outdoor temperature is outside these limits, specimens should not be outdoors for long times. In extreme high temperatures, consider use of a cold pack. 2. 2° C -10° C: Use ice or ice packs in container between clinic and lab.		

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2. http://iweb.lati.tec.sd.us
3. http://www.clinlab.com/docs/specimen
4. Diagnostic procedures, CMC, Vellore, India.
5. Lennette HE, Balows A, Hauser WJ et al. Collection, Handling and Processing of Specimen. In Manual of Clinical Microbiology, 4th Ed, ASM, Washington, DC, 73-98, 1985.
6. HPTN 052 SSP Manual DRAFT Version 2.0 or current version

Appendix A: Specimen Summary

SPECIMEN TYPE	ASSOCIATED LAB TESTS	COMMENTS

Venous Blood
• Plain, no additive
  o Serum
• Anticoagulant
  o Whole Blood
  o Plasma
	• Biochemistry
	• Hematology
	• HIV Viral Load
	• Genotyping
	• Serology
	  o HIV
	  o Hepatitis B
	  o Syphilis testing
	• CD4/CD8 profile
	• PBMC
	• Blood culture
		Only trained staff should perform phlebotomy procedures. Many tests (such as lactate) have special handling and transportation requirements. Verify all test orders before obtaining sample.

Fingerstick Blood	• Malarial Thick and thin smears	Slides are prepared at the site of collection and sent to laboratory for staining.
Urine	• Pregnancy Testing	Specimens are collected by the patient with

	• Microbiology • Urinalysis • NAA for GC/CT	supervision of clinic staff.
Cerebral Spinal Fluid (CSF)	• Microbiology • Biochemistry • Hematology	Collected by physician
Vaginal Swabs	• Wet Mount for BV	Collected by physician
Endocervical swabs	• NAA for GC/CT • HIV-1 RNA Viral Load	Collected by physician
Pleural Fluid	• Microbiology	Collected by physician
Sputum	• Microbiology	Collected by physician, clinic staff, or patient under supervision of study staff
Semen	• HIV-1 RNA Viral Load	Specimens are collected by the patient with supervision of clinic staff
Genital Ulcer Swab	• Multiplex PCR (JHU)	Collected by physician
Feces	• Microbiology	Specimens are collected by the patient with supervision of clinic staff
Skin lesion exudates	• Fungal wet mount	Collected by physician or clinic staff

Procedure: SAMPLE DESTRUCTION

Prepared by:	Date Adopted:

Review Date:	Revision Date:	Signature:

INTENDED USE

This policy outlines laboratory procedure for the destruction of stored samples in the lab's custody following instructions from the owners of the samples or the responsible PI as is applicable.

SUMMARY AND BACKGROUND

The _____ Laboratory receives samples from studies conducted locally and elsewhere. These studies may be HIV from Adult and Pediatric AIDS Clinical Trials (ACTG) studies, HIV Prevention Trials Network (HPTN) studies, _____ studies or other local and international studies. The Laboratory has stored samples for local and cooperative studies. Some of these samples may be sent out to other laboratories in and outside Malawi for other required testing as mandated by the respective protocols.

At the completion of a study, when there are specimens still being stored on-site, a determination is made by the sponsor(s) of the study or the Principal Investigator(s) to destroy or continue to store the specimens in question. All hospital and /or university policies are to be followed when handling or discarding specimens.

For older studies, the executive group may make a determination to destroy or continue to store the specimens in question. All hospital and / or university policies are to be followed when handling or discarding specimens.

SAMPLE DESTRUCTION

- The laboratory will be notified by the specific entity, either study team(s) or Principal Investigator if specimens need to be destroyed. This authority will also dictate *exactly* which samples are to be destroyed.

- Laboratory staff will fill in on the "Destruction of _____Samples Table" the following information: date & time of destruction, protocol number, notifying authority, the nature of the samples being destroyed, Laboratory staff member's signature & date and the Laboratory Director or designee's signature & date. These records will be kept in the appropriate study folder.

- Confirmation of destruction will be sent out as requested by the study sponsor(s) or Principal Investigator.

- The notifying authority will provide the laboratory with a date by which the specimens need to be destroyed. This notification may also include any special requirements for destruction or documentation.

- All liquid nitrogen samples will be taken out of the LN_2 freezer and placed in the –70°C freezer overnight. The next day they will be placed into the biohazard boxes/bags. Specimens stored in the –70°C freezer will be thrown directly into the biohazard box/bag.

- The biohazard boxes/bags will be autoclaved for sterilization and sent to the hospital incinerator for destruction in accordance with _____policy.

- Specimen inventories will be checked before destruction. Any discrepancies will be noted.

- Specimens will be removed from specimen storage section of the LDMS.

- FSTRF will be asked to help complete the above task.

- FSTRF will be asked to run a patch so that comments are made in the specimen management section about the destruction of the samples along with the date.

- Copies of the storage reports will be kept along with the Destruction of _____Samples Table.

Destruction of _____samples table

Date & Time	_____*protocol*	*Who notified us?*	*Which specimens were destroyed?*

Laboratory Staff member's signature & date: _____

PI's signature & date: _____

REFERENCES

1) HPTN Central Lab Policy: Destruction or Research Laboratory Specimens

LDMS MANAGEMENT		
Title: LDMS Management	SOP Number:	Total Number of Pages:
Effective Date:	Supersedes:	Primary Author: S.Hanas
Revision History: 20 Apr, 2 Aug, 25 Nov 2004		
Reviewed by: Dr. P. Balakrishnan, E. Livant		Review Date:
Approval Signature and Date:		
Distributed to: All HPTN 052 Lab & Admn. members		Distribution Date:

PURPOSE

The Laboratory Data Management System (LDMS) is a window based program using both graphic symbols and drop down menus. It was created by the Frontier Science and Technology Research Foundation (FSTRF). It helps in managing data systems in the laboratory and simplifies the process of finding specimens stored in the lab and retrieving relevant specimen information. It is also used in the process of shipping specimens to other laboratories.

SCOPE

This procedure applies to all HIV Prevention Trials Network (HPTN) LDMS functions to be performed in YRG CARE laboratories. At this time, only HPTN or Adult AIDS Clinical Trials Group (AACTG) samples should be stored in LDMS. For more specific instructions, please refer to the SSP manual for HPTN 052, the LDMS user manual or the Frontier Science and Technology Research Foundation (FSTRF) website: http://www.fstrf.org (access codes required to view the HPTN information links).

Only trained users who have been authorized should use the LDMS system. This SOP is designed as a guide but does not replace appropriate instruction.

TECHNICAL ASSISTANCE

Most questions can be answered by using the LDMS user's manual. This should always be consulted first. The YRG CARE Information Technology (IT) department should be notified of all

problems which cannot be resolved by laboratory staff and should be consulted before contacting FSTRF.

For problems which cannot be resolved locally, contact FSTRF. Specific FSTRF staff may be contacted but it is advisable to always copy hptn.ldms.usersupport@fstrf.org on all emails to ensure prompt reply. You should include all pertinent information such as your name, your site and site number, the nature of the problem and steps already taken to fix the problem. Once a communication on a problem has been started, you do not need to repeat all of this information. FSTRF will usually respond within 24 hours including weekends. Every effort should be made to resolve problems before contacting technical support.

PRE-LDMS SPECIMEN PROCESSING

Types

Specimen types will vary based on study requirements. A list of specimen types by study can be located at www.fstrf.org → LDMS → HPTN/HVTN → REFERENCE GUIDES →HPTN →Specimen Management Entry Guides. For HPTN 052, the following specimens may be stored: serum, plasma, Peripheral Blood Mononuclear Cells (PBMC), Genital secretions (female and male) and Genital Ulcer swabs for Multiplex PCR (See table 10-3 from the SSP manual).

Verification

When the specimens are received at the local LDMS Laboratory, each specimen delivery will be checked to ensure that the all label information and that the type and number of specimens marked on the Laboratory Investigation Request Cum Specimen Tracking Sheet is correct. If discrepancies are noticed, the clinic staff should be contacted and local procedures for QC and correction followed.

LDMS PROCEDURES

Entry of details from tracking sheet

The LDMS laboratory technologist will then open the LDMS Specimen Management window and position the cursor at the Group box.

1. The technologist will hand enter

a. The group

b. PTID

c. Protocol

d. Visit code

e. Specimen collection date

f. Specimen receipt date

g. Recd time

h. # of tubes

i. Primary type

2. The add button will be pressed to save the information.

3. Each type of specimen will then be logged in individually and then aliquots generated for that specimen type.

    a. # of aliquots

    b. Volume

    c. Units

    d. Derivative and sub derivative

4. The add button will be pressed to save the information.

5. Press the Red Cross button to save the changes.

6. After saving changes, mark the record number displayed on the bottom of the specimen tracking sheet.

7. LDMS will ask if you want to print labels. Usually, you will answer no and perform this procedure when all specimens have been entered.

Label generation

If the specimen is to be aliquoted and stored, LDMS-generated labels will be prepared using the LDMS system and printed on the LDMS printer using label stock certified for long term freezer storage and dry ice shipping.

1. On the main tool bar, select tools, then labels.
2. Select the group.
3. Select the format: ACTG1 for ACTG or LDMS1 for HPTN.
4. Perform a search for the specific labels by using Spec ID and/or fields.
    a. In order to generate the correct labels, you can select different criteria necessary: PID, additive, date, etc… Multiple criteria can be entered as needed.
5. If using a sheet of labels that has already been used, enter the number of labels missing in the "skip" field to start at the first label remaining.
6. Press the execute key and review the labels. Arrange the labels sheet in the printer and print. Use caution when re using label sheets so that labels do not dislodge and become stuck in the printer. Remove labels which are not well placed before printing.

Storage

1. Go to the storage module.
2. Specimens can be located using either the search tab or the bulk add tab. Generally, the bulk add tab is used.
    a. If no criteria is entered and the execute button is hit, all specimens which have been entered but not stored will appear in results grid. Otherwise enter criteria to search for specific specimens.

b. *Note: If an aliquot is stored and then that storage is deleted, LDMS will still consider the specimen as stored. Return to the specimen management module and locate that specific aliquot. Right click on the aliquot and select "never stored". The aliquot may now be stored.*

c. Drag specimens to the appropriate storage containers on the left grid. If using 9X9 boxes and entering multiple aliquots you may choose auto all to enter these. *Do not use this function if entering PBMC specimens because these must be arranged to specific* locations *per the PBMC box map posted*

d. You may select "do not ask again" when asked to modify volume unless this is desired.

e. Print out storage reports to accompany the labels so that the specimens can be stored.

Correcting Specimen Information

To correct specimen information, Go to Tasks Specimen Mgt on the menu bar or click the Specimen Mgt button on the tool bar.

1. Use the browse button or extended search button on the tool bar to retrieve the specimen information on the Entry screen.
    a. Enter a Group, Type and ID in their respective boxes, and then click the Run button to display all specimen information that matches the selected criteria.
    b. Highlight the record to correct then click the Select button.
2. On the Entry screen, change any information on the screen except the specimen ID number.
3. Click on the Primary Details button to enter the changes you made. Enter any changes in the comment field. For Example: "Date changed from02 NOV 03 to 02 NOV 04 by CPM 29 NOV 04".
4. Click on the Save button on the LDMS toolbar to save the changes to the specimen record.

Moving Specimen in the Storage

1. Go to Tasks-Storage Mgt. or click the Storage Mgt button on LDMS toolbar.
2. Click on the Move tab.
3. Click on the specimen in the storage structure list box on the left side of the screen.

4. Hold the mouse button down and drag the specimen to a container in the destination list box on the right side of the screen. When an outline appears around the appropriate destination container, let go of the mouse button.
5. Verify the specimen you want to move then click the Yes button on the storage dialog box.
6. The specimen position selection box will appear, select the position you want to put your specimen then click Put Here button.
7. A success dialog box will appear listing the position to which the specimen was added. Make note of the new specimen position, and then click the OK button on the success dialog box.

Printing Reports

1. To print a box report click on the Main View tab.
2. Highlight the box you want to print report.
3. Click the Details button on the bottom left side of the Main View screen.
4. The Details dialog box will appear, click on the Box Report button.
5. The box report will appear, click the print button on the toolbar.

Data Back Up

Data back up should be done as needed and after any large input of data. **IF THERE IS A SYSTEM CRASH ALL DATA THAT HAS NOT BEEN BACKED UP WILL BE LOST AND WILL NEED TO BE REENTERED AFTER SYSTEM RECOVERY.** Thus it is the responsibility of LDMS technicians to back up the data after data entry.

1. Find the FSTRF folder on the C drive (Look in the "My Computer" icon.) This folder (or the tables and assay folders within) must be copied to another location such as the lab server, a CD, another desktop, or a tape drive.
2. It is recommended that a backup be kept in a different YRG CARE building in case of fire.
3. Record the transaction on the log sheet.
4. It is recommended that 2 backups always be in use in case one fails. These backups must be in a separate location away from the primary LDMS computer.
5. In case of computer crash, contact the IT department.

Exporting Data

Data should be exported on a weekly basis to FSTRF. **Exporting data does not replace backing up data in the event of a computer crash.** The first time you export you may need to contact FSTRF support to set up the export module. In order to export:

1. Ensure there is an internet connection.
2. Go to Tasks on the tool bar then click on Export.
3. Click on "Exp+Trans" button.
4. The process may take several minutes and may not show activity. Do not perform any other activities on the computer until LDMS displays a message saying the export was completed.
5. There will be a record of the export displayed in the transferred files grid to confirm the export.
6. In case any files remain in the top grid, note the names of these files and contact FSTRF for assistance.
7. After a successful export, record the transaction on the log sheet.

Shipping

This section covers only LDMS procedures related to shipping specimens. See the following section for more detailed crucial information related to shipping specimens.

You must first create a shipping batch. Shipping batches may be created by searching for individual specimens or by shipping entire containers.

1. Searching for individual specimens
    a. Enter the shipping module. Go to Tasks-Shipping on the Menu bar or click the Shipping button on the toolbar.
    b. Click on the Shipment Destination tab. Locate the LDMS address or enter a non-LDMS address.
    c. Configure the dimension (rows by columns) and order of the shipping box.
    d. Click on the Setup Shipment tab.
    e. Enter HPTN for group. Enter the search criteria in the type and ID combo boxes. Move the query into the search grid by clicking on the arrow.
    f. This may be repeated for multiple criteria.
    g. Click on the Execute button to search for specimens.

h. Select the desired specimens to be shipped.
i. Click on the Add button to create the batch.

2. Shipping entire containers
    a. In the Storage Module, select the box to be shipped.
    b. Click on the Details button.
    c. Click on the Mark to Ship button. DO NOT click the checkmark for Sort box by Protocol.
    d. Enter the Shipping Module.
    e. Follow the same procedure as above but select "Marked Boxes" from the Type combo box.
    f. The ID will only show boxes that have been marked for shipment.
    g. Select the desired box.

3. Once a shipping batch is created you must create a shipping Manifest. Highlight the desired batch and click on the "Manifest Report" button. Print this report.

4. Click on the "Shipping Box Report" button. Print this report and verify that the information on the report matches the actual shipping container.

5. To create a shipping disc, right click on the highlighted batch and select the "Create Shipping Disc" option. Follow the instructions on the screen to create the shipping file. This file may be sent electronically to the destination or included on floppy.

6. You are now ready to ship. Include the Shipping manifest, Box reports and shipping disc with your shipment.

POST LDMS SHIPPING PROCEDURES

In Country Shipping

In country shipment does not require any permit from State or Federal Government. The document needed is a letter declaring the commercial value, nature of the goods, purpose of shipment, and storage condition of the shipment.

Plasma (for HIV Genotyping) will be shipped to National AIDS Research Institute (NARI), Pune, India. The specimens to be tested will be batched (as per JHU CL instructions) and shipped on a monthly basis to the regional laboratory (NARI) through World Courier. Technical Facilitator will track the specimens using LDMS software and retrieve the specimens from the freezer storage. The specimens will be arranged in a cryobox, (use rubber bands over the cryobox so the lid may not open during the shipment). Then the cryobox is placed in a polythene seal able Biohazard SafTPak (STP 711- can hold only one cryo box) and sealed. Then STP 711 is placed in a thermocol box filled with dry ice and, covered and then placed in a carton box and sealed, Tech. Facilitator will label the carton box with Dry ice specification, shipper address, consignee address, UN Number for specimen for the item shipped and Dry ice, 24 hrs contact name, address and phone number. Technical Facilitator will create an electronic shipping manifest for each shipment using the LDMS system. The electronic manifest disk will be placed in the shipping container along with the shipping manifest as hard copy. After finishing the process, the box is handed over to World Courier people for further shipment process. World courier issue a House Airway Bill (HAWB), HAWB number is useful in tracking the shipment status and HAWB number will be intimated to the Consignee through email. Once the shipment is lifted, the responsible person (at NARI) would be intimated about the shipment through telephone also.

International shipping

The designated specimens for Quality assurance (10% of study specimens as per the instructions given by JHU CL) and the specimens for special tests (Cervical swab for HIV-1 RNA, Semen for HIV-1 RNA and Genital ulcer - multiplex PCR) are shipped to HPTN Central laboratory, USA.

The Technical Facilitator will retrieve the designated specimens from the freezer storage based on the storage report generated by LDMS. The Technical Facilitator arrange the specimens in a a

cryobox, (use rubber bands over the cryobox so the lid may not open during shipment). Then the cryobox is placed in a polythene seal able Biohazard SafTPak (STP 711- can hold only one cryo box) and sealed. Then STP 711 is placed in a thermocol box, fill with dry ice and covered, placed in a carton box and sealed, Tech Facilitator will label the carton box with Dry ice specification, shipper address, consignee address, UN Number for specimen and Dry ice, 24 hrs contact name, address and phone number. Technical Facilitator will create an electronic shipping manifest for each shipment by the LDMS system. The electronic manifest disk will be placed in the shipping container along with the shipping manifest and box report prior to final packing and pickup. After finishing the process, the box is handed over to World Courier people for shipment. World courier issue a House Airway Bill (HAWB), HAWB number is useful in tracking the shipment status. Once the shipment is lifted, the responsible person (coordinator) in HPTN Central Lab should be intimated about the shipment and HAWB number will be emailed.

The following documents should be produced while exporting bio specimens to USA:

1. Export permit from the Directorate General of Foreign Trade, India.
2. 3 copies of Commercial Invoice
3. 3 Copies of Packing list
4. A letter declaring the nature of the consignment, storage and transporting temperature.
5. CDC permit in the name of the consigner.

Labeling Of The Shipping Box:

As the specimens are exported for testing and quality assurance purpose, the shipment may be declared as Diagnostic specimens. In this case, each package and overpack should be labeled with each of the following

a. “DIAGNOSTIC SPECIMENS PACKED IN COMPLIANCE WITH IATA PACKAGING INSTRUCTION 650”.
b. The full NAME AND ADDRESS of the shipper and the consignee.

c. Net weight of Dry Ice used, the proper shipping name (“Carbon dioxide, solid” or “dry ice”) and the UN Number (UN 1845).

Note: The net weight of the Diagnostic specimens contained in a single package should not exceed 4L or 4Kg.

REFERENCES:

1. HPTN 052 SSP Manual, version 0.92.

2. http://www.hptn.org/hptn_structure/ldms.htm

3. LDMS User’s manual, current version

Appendix A: LDMS Specimen Management Guide to Logging in 052 Specimens

Test	Primary	Additive	Derivative	Sub Add/Deriv	Primary Volume	Aliquot Volume
Serum Storage	BLD	NON	SER	N/A	10 ml	1 ml
Plasma Storage	BLD	EDT	PL2	N/A	10 ml	1 ml
PBMC Storage	BLD	EDT	CEL	DMS	30 ml	10 million/ 1ml
Genital Secretions (Female)	CER	NON	SNO	GIT	24 µl	24 µl
Genital Secretions (Male)	SEM	NON	FLD	N/A	0.3 ml	0.3 ml
Swab for Multiplex	GLU	NON	SWB	N/A	100 µl	100 µl
Whole Blood Storage	BLD	EDT	BLD	N/A	5 ml	1 ml

8.6.2 Cervico-vaginal Lavage

All female participants in cohorts C and D will have Cervico-vaginal Lavage (CVL) collected to measure genital secretion HIV viral load at each scheduled study visit. CVL will also be tested for HIV resistance patterns at baseline and Day 14.

8.6.2.1 Specimen Collection

1. Draw up 10 mL of sterile, isotonic, nonbacteriostatic normal saline through a needle attached to a 10-mL syringe. Remove the needle from the syringe, and then place the pipette on the syringe. (The pipette is cut below the bulb and the remaining pipette tip is placed on the end of a 10-mL syringe.)

2. With the speculum in place, introduce the pipette into the vagina.

3. Expel the saline to bathe the cervix (pipette should be directed towards the cervical os).

4. Allow the fluid to pool into the posterior fornix, and aspirate with the tip of the pipette against the inner surface of the posterior blade of the speculum into the same syringe. Avoid contact between the tip of the pipette and the epithelium.

5. Repeat this procedure 4 times with the same fluid; do not add any additional saline to the specimen.

6. Aspirate the fluid a final time and place in a labeled [small SCHARP participant ID and small SCHARP date label] 15-mL plastic (conical) centrifuge tube. The tube should also be labeled to indicate origin: cervicovaginal lavage fluid.

8.6.2.2 Specimen Processing

- Specimens should be placed on ice immediately and transported to the laboratory or refrigerated.

- Log specimens in the LDMS and generate LDMS labels.

- Centrifuge the collected fluid at 600 x g for 10 minutes.

- Remove the supernatant, make as many 1mL aliquots as possible using labeled [LDMS generated label] cryovials and freeze as soon as possible at -70° C until shipped.

- Resuspend the cell pellet in 0.5 mL of 1X phosphate-buffered saline.

- Transfer the resuspended pellet into a labeled [LDMS generated label] 2.0 mL cryovial and freeze at -70° C.

8.6.2.3 Specimen Shipping

Specimens (two 1mL aliquots of supernatant and one resuspended cell pellet) can be batched and shipped on dry ice to the CL at the end of the study.

Temporarily store any extra frozen supernatant.

⟶ Do not ship on Thursday, Friday or the day before a holiday. Please call the laboratory around holiday times to check available days for shipping.

⟶ Ship Priority Overnight to ensure samples arrive at the lab the next morning.
Include LDMS shipping manifest, box report and diskette with specimens.

Once the CL has advised that the samples have been successfully analyzed, temporarily stored extra supernatant samples are to be destroyed according to local procedures, and deleted from the LDMS.

10.5.4 Semen

Semen will be collected for specimen storage and to test for seminal HIV viral load.

Prior to semen collection, all semen collection containers should be labeled (participant ID label and hand-written date). If at all possible, semen specimens should be collected in the clinic; however, if this option is not feasible, participants may collect this specimen at home and return it to the clinic within two hours of collection. If the sample is collected at home, participants should be given the labeled semen collection container and instructions for how to collect and return the specimen prior to collection. Semen should be collected as follows:

- Provide participant with a labeled semen collection container.
- Instruct participant to wash his hands and penis and then use an antiseptic towelette to wipe the head of the penis including the opening. If the participant is uncircumcised, ask him to pull back the foreskin before cleaning the head and opening.
- Provide the participant with a private location and instruct him to masturbate, collecting the entire specimen in the container. If the specimen is collected at home, the specimen should be returned to the clinic within 2 hours.

Semen samples must be processed by the lab within 4 hours of specimen collection (refer to Section 10.6.5).

10.5.5 Cervical/Vaginal Secretions

If the woman is menstruating during a visit that requires a pelvic exam, she may return before the visit window closes or for an interim visit during which the exam can be conducted and the samples collected. If this is not possible, the exam and sample collection can be conducted at the next monthly visit. If the samples are still unattainable at this subsequent monthly visit (either within the visit window or at an interim visit), study staff should not attempt to collect the samples again until the protocol requires it. Study staff should try to schedule the enrollment visit at a time when the female index case is not menstruating as this is a critical baseline measurement that should be collected prior to ART initiation.

10.5.5.1 Samples for Cervical HIV-1 RNA

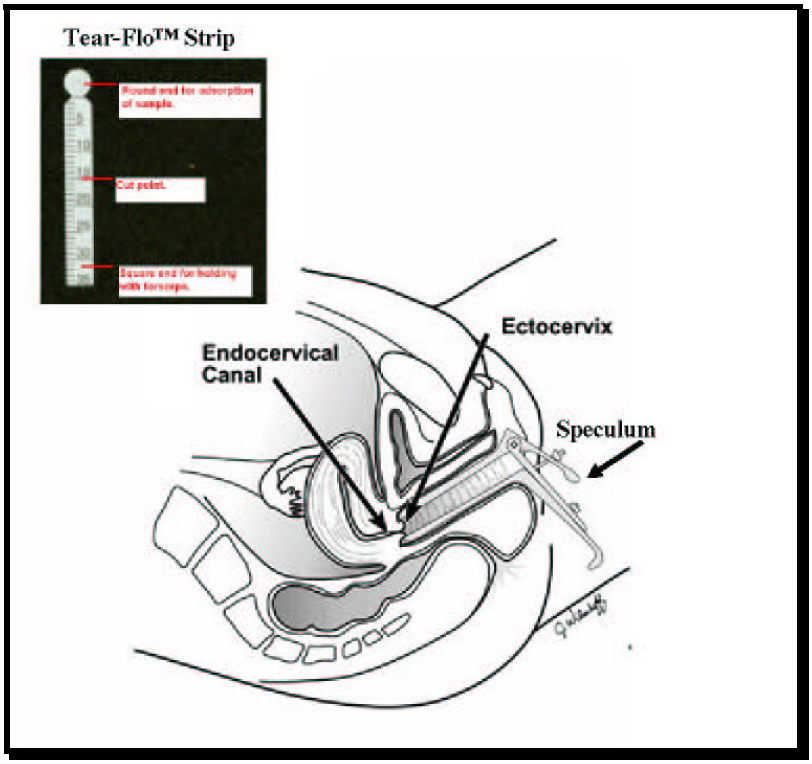
Cervical secretions will be collected for specimen storage and to test for cervical HIV viral load. Table 10-5 depicts when cervical secretion samples should be collected. This specimen should be collected before any other cervical or vaginal samples.

The participant must refrain from any kind of sexual activity, douching, and inserting any intravaginal products for at least 48 hours prior to the collection of cervical specimens.

Tear-Flo™ will be used as wicks to collect primarily cell-free virions from the endocervical canal fluid in the following manner (refer to Figure 10-2):

- Help the participant assume the lithotomy position.
- Gently insert an unlubricated and appropriately-sized speculum into the vagina and lock it into place.
- Use a large cotton-tipped swab to gently remove excessive mucas or menses clots in the vagina and the cervical os before inserting the Tear-Flo™ strips.
- Use forceps (ring or sponge forceps work well) to hold the strip on the squared end of the strip and gently insert **two** Tear-Flo™ strips simultaneously into the vagina, place through the cervical os into the distal endocervical canal and hold in place to adsorb sample. Each Tear-Flo™ strip adsorbs approximately 12µl of specimen. Adsorption usually takes approximately one minute, but may take a little longer.
- Hold the round end of the two strips over and slightly inside one labeled plastic transport tube (1.5 or 2.0-mL cryovial) containing 500 µL of NASBA 1x Nucleic Acid Sequence Based Amplification (NASBA) lysis buffer. Cut the strips at the "15" mark with scissors, allowing the round end to fall into the cryovial tube containing buffer. Cap and invert the sample or vortex it for 5 seconds.
  Note: If the Tear-Flo™ strips are to be stored in NASBA lysis buffer, the buffer must be crystal free before you begin. Most crystals will dissolve by placing the lysis buffer tube at room temperature for a few hours. If crystals remain in the tube, it should be vortexed until the crystals are gone.
- Two sets of specimens (2 Tear-Flo™ strips each) should be collected: one for specimen storage and the other for cervical HIV viral load.
- Tear-Flo™ strips in NASBA are stable for 24 hours at room temperature (~25°C), for 14 days at 2-8°C, and one year at -70°C. Keep the sample refrigerated or on ice until it is frozen at -70°C. DO NOT store specimens in lysis buffer at -20°C.

Figure 10-2: Using Tear-Flo™ Strips to Collect Cervical Secretions.



Section 4: Documentation

1. Documentation Standards

All documents which will be retained for official records must meet certain standards. All entries must be legible and contain the identification of the person who made them and the date they were made. Hand written entries must be made in black or blue ink unless otherwise noted in an SOP. Documents must be retained for predefined periods of time (e.g. : 2 years, 5 years, 2 years after the completion of a study, indefinitely or until permission is obtained from DAIDS).

2. Error Correction

It is expected that errors will be made when writing documents. When correcting these errors, it is important to follow a specific format. A single line is drawn through the original entry so that it is still legible. Never use whiteout or deface an entry so that it can not be read. When no explanation is necessary, the new information can simply be written next to the original entry. Every correction must have the initials/signature and date of the person who made the correction. When the correction is not self-explanatory, a comment must be added. These comments can be written on the same page if there is adequate space. The other side of the page and additional pages may be used but it must be clear to what the comments refer.

3. Results Reporting

Laboratory results must include the following elements:

a. Laboratory Name
b. Laboratory Address
c. Laboratory phone number
d. Specimen collection date and time
e. Name of test(s)
f. Units (when applicable)
g. Normal Range (when applicable)
h. Reporting date
i. Comments as needed
j. Performing technologist

Erroneous results

a. Any time an error is discovered or strongly suspected, notify a laboratory manager or designee immediately.
b. The laboratory manager or designee must notify the staff in the clinical unit immediately. If possible, notify the physician who handles that patient.
c. If the correct result can be given, it must be made on all the appropriate forms where the result appears using proper documentation standards.
d. The final copy must be corrected if possible. If not, a new copy should be attached to the original final copy. The corrections should include necessary information describing the error.

e. If it is not possible to definitively ascertain the correct result, the result should be stricken in all places it appears using proper documentation standards with comments. Notify the clinical unit and allow them to decide if they wish to attempt to obtain a new sample.
f. Laboratory errors must be tracked as part of the laboratory Quality Assurance-Improvement plan. Serious errors require specific investigation and follow up.

4. Source Documents

Every laboratory result must be traceable to a "source document". The source document is the first place a result is recorded. The source document should be defined for each test done in the lab and a system in place for filing and retrieval of these documents.

a. Printouts
b. Log books/worksheets or registers
    i. This is ideal for tests such as HIV rapid tests, urinalysis or wet mounts where there is no printed document from an analyzer. It is important in these cases that there is all the required information in the log book.
c. Electronic
    i. Electronic source documents must be backed up in a separate location. The laboratory must maintain the software to read the document for the duration of the time the laboratory would retain a hard copy.
    ii. When it is possible to maintain electronic source documents, this is preferred.
d. Thermal paper
    i. Thermal paper printouts are known to fade and thus do not make the ideal source document. In these cases, the laboratory may define a log book as the source document or make a "certified copy" of the thermal printout:
        1. Photocopy the thermal printout.
        2. On the photocopy write or stamp "I certify this copy is an unaltered version of the original"
        3. Initial (or sign) and date that statement.
        4. Attach the thermal copy to the certified copy.

5. Communications to Network Laboratory (NL)

The HPTN/MTN NL needs to be informed as quickly as possible of any serious instrument problems/failures, QC and reagent problems. The NL need to be made aware whenever your site puts in place any back up measures (such as using back-up laboratories and equipment or other sites' laboratories), switches in reagents or kits, or updates normal ranges or toxicity grading based on a change of laboratory/instrument, reagent, or kit.

SCHARP has QC checks in place that may need to be amended based on this information. Please inform the NL, SCHARP, and FHI within 24 hours of any such changes and provide the start dates of these measures to ensure that all necessary QC checks can be updated.

We would like to remind all sites that some reagent changes may require re-evaluation of your site's normal ranges and toxicity grading. If you have any questions regarding a normal range change, please contact the HPTN NL (centrallab@hptn.org) and or the MTN NL (mtncentrallab@mtnstopshiv.org).

6. Notes to File

Events or issues may arise that require specific documentation to be filed for further reference. The filing of these is at the discretion of the laboratory but they must be kept in such a manner so that they can be easily retrieved.

Examples (partial list) of Notes to files:

a. Deviation from laboratory sops
b. Deviation from SSP – may constitute a protocol event form (see attached document)
c. Deviation from required documentation practices such as second signature missing from HIV readings
d. QC failures – QC has been out of range, resulting in a delay of reporting results
e. Reagent issues – ran out of reagents or supplies needed to perform the required protocol testing
f. LDMS issues – if any major downtime on the LDMS where manual labels are made or if errors in data entry are found
g. If samples are sent to another laboratory to be analyzed, the time period needs to be indicated on the form
h. Instrument Failures/down times– refrigerator or freezer temperature problems, repairs needed on instruments.
i. Technical Errors – laboratory personnel made a technical error
j. Personal changes in the laboratory – adding new technicians or removal of technicians

7. Protocol Events

Serious problems relating to study execution must be documented and communicated to the network. These are cases which add extra risk to the participants, do not follow protocol guidelines or GCLP. Examples of this for the laboratory include but are not limited to;

a. Repeated QC failures/ instrument test method failures
b. Required tests not being performed repeatedly
c. Numerous specimens not stored
d. Incorrect results being released, specifically HIV results

If you suspect you have a protocol event, contact the NL. They will instruct you if you need to complete a Protocol Event form.

PROTOCOL EVENT FORM

Use this form to report Protocol deviations that are defined as Protocol Events according to Policy Number 015. This form is to be used by staff at the HPTU sites, CORE, SDMC, and CL. Upon completion, email this form to the distribution list created for the study.

EVENT INFORMATION			
DAIDS Site Number:	***Your Site number***	Date event occurred	
		Date of site awareness	
Protocol Number		Date event reported	
Participant ID ***(if applicable)***	***Do not fill in if study is blinded***	Was event reported to the IRB/IEC?	☒ ***yes*** ☐ ***no***
Report Completed By:	***[Name and title]***		
	Contact phone and/or email:		

BRIEF SUMMARY OF EVENT (Description of event, location it occurred if relevant)

(Maintain all documentation in study files.)

For a participant who has a positive HIV rapid test, this sample is sent for 1st Western Blot; then a subsequent blood sample is collected for the 2nd WB test. This second blood sample should be used for RPR testing in the case of a seroconversion exit visit from protocol XXX

The study staff did not use, the second WB sample collected on xx-XXX-xx to perform the RPR test; but instead RPR sample was collected and tested on xx-XXX-XX during her Exit vist from protocol xxx study

What steps have been taken to address this event?
The relevant study staff have been re-trained on the exit visit procedure for those who seroconvert.

What steps have been taken to prevent further occurrences? Same as above.

Regulatory Note to File
Site Name
Lab Name

Date Submitted:	Date that note is sent to everyone on the copy list
Submitted by:	Lab Supervisor/Lab Manager/Lab Director or Lab QA coordinator
Occurrence/Issue:	State the nature of the occurrence/issue, giving enough details to make the occurrence/issue understandable to everyone reading the document.
Dates of Occurrence/issue:	Period of time of the occurrence/issue took place
Date lab notified:	Date that the lab management staff became aware of occurrence/issue
PTIDs involved:	Note any patient IDs
Resolution:	State clearly how the occurrence/issue was resolved
Date of Resolution	Date that the resolution occurred
Corrective Action:	State any corrective action that was needed in addition to the resolution. This would include retraining of staff or any actions put in place so the problem does not reoccur
Copies to:	Lab Protocol File Lab personnel file (if lab staff involved in retraining) Site study coordinator Site protocol regulatory file FHI protocol HPTN CL SCHARP

Examples of Notes to files:

1. Deviation from lab sops
2. Deviation from SSP – may constitute a protocol event form (see attached document)
3. Deviation from required documentation practices such as second signature missing from HIV readings
4. QC failures – QC has been out of range, resulting in a delay of reporting results
5. Reagent issues – ran of reagents or supplies needed to perform the required protocol testing
6. LDMS issues – if any major downtime on the LDMS where manual labels are made or if errors in data entry are found
7. If samples are sent to another lab to be run to be performed, the time period needs to be indicated on the form
8. Instrument Failures/down times– refrigerator or freezer temperature problems, repairs needed on instruments.
9. Technical Errors – lab personnel made a technical error
10. Personal changes in the laboratory – adding new techs or removal of techs

Regulatory Note to File
Johns Hopkins University
HIV Specialty Lab

Date Submitted: April 6, 2006
Submitted by: Estelle Piwowar-Manning, MT(ASCP)
HPTN CL Deputy Director

Occurrence/Issue: HIV QA samples were performed on protocol samples from Iceland. The external HIV control was out of range. The kit controls were acceptable. The technologist released the results. On review of the run, the out of range control was noted by the lab supervisor, who in turn informed the HPTN CL.

Dates of Occurrence/issue: April 1, 2006

Date lab notified: April 3, 2006

PTIDs involved: xxx-000001-1
xxx-000002-2
xxx-000003-3

Resolution: The HIV run was repeated on April 4, 2006. The external control was in range as were the kit controls. The results were released/amended. All results were comparable to the previous run.

Date of Resolution April 4, 2006

Corrective Action: The HIV lab tech involved in the incident was counseled. Retraining in HIV EIA assay took place on April 3, 2006. The lab put into place a peer review of the runs if a supervisor is not present. The HIV EIA sop was modified on April 5, 2006 to include peer review.

Copies to: Lab Protocol File
Lab personnel file (if lab staff involved in retraining)
Site study coordinator
Site protocol regulatory file
FHI protocol
HPTN CL
SCHARP

Section 5: Method Validation

New instruments or methods must be validated onsite to ensure that they perform in accordance with manufacturer's claims, are appropriate for different settings and that site staff have an adequate level of competence. Modifications to existing procedures and deviations from manufacturer's directions are also subject to validation studies.

Validation requirements vary depending on if the instrument or method is FDA approved. Quantitative and qualitative methods will have different types of validations.

Laboratories are not required to verify or establish performance specifications for any test system used by the laboratory before April 24, 2003 (per CLIA 42 CFR part 493.1253) if they have documented performance of these systems through Internal Quality Control and External Proficiency testing.

Validation of Normal Ranges is Essential! Please ensure that the normal ranges for quantitative tests at your site have been validated or that a full study has been conducted and approved.

The following information in this section describes method validation.

Procedure: Quantitative Assay Validation Procedure Version 1.0

Prepared by	Date Adopted	Supersedes Procedure #
Paul Richardson		NEW

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies
HPTN Laboratories			

VALIDATION STUDIES

Any time an instrument or methodology is changed within the laboratory, validation studies must be performed. Documentation of these studies must be maintained for the life of the instrument or methodology. Results of these studies must verify the performance specifications and claims of the manufacturer.

The following describes assay validation studies suitable for manual and automated quantitative assays such as for chemistry and hematology.

If these procedures do not appear suitable for your assays please contact the HPTN Central Laboratory (CentralLab@HPTN.org) for clarification.

Results of assay validation studies must be sent to the HPTN Central Laboratory for approval before that assay can be used in a HPTN protocol.
(email CentralLab@HPTN.org)

Studies for quantitative assays that are FDA approved and unmodified must contain the following:

1. **Accuracy**
   Is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
   Determined by:
   a. Assay materials with assigned values
   b. Comparing patient specimen results with a method of long standing use
   c. Verifying results from inter-laboratory survey specimens
   d. Splitting specimens with another sufficiently accredited laboratory.

   Results must demonstrate the system is accurate enough to provide clinically valid patient results. Limits of acceptability should be set by the Laboratory Director.

2. **Precision**
   Precision is reproducibility, the agreement of the measurements of replicate runs of the same sample.

   Precision is the process of determining the range of random errors. The precision is measured in terms of coefficient of variation (CV) and standard deviation (SD). The smaller the CV and SD, the better the precision.
   This can be determined by running a minimum of 20 replicates of a specimen or QC material over a period of 10 to 20 days if possible. The mean, CV and SD can all be calculated from the data obtained.

   Precision data must demonstrate the assay performance, which is comparable to the performance specifications published by the manufacturer. When there are no specifications published, limits of acceptability must be set by the Laboratory Director.

3. **Verification of Reportable Range.**

   This is the range of test values over which there is a valid relationship of the instrument, kit or test systems measurement response. The response may not necessarily be linear.
   a. The laboratory must demonstrate a relationship between the actual and expected values of a test procedure
   b. Verification must be run for assay validation and at a minimum annually
   c. Verification determines the lower limit of reporting as well as the upper limit of reporting
   d. plot the expected values on the x axis and the actual values on the y axis
   e. Manufacturer claims must be verified.

f. If the reportable range study indicates a usable range outside the limits indicated by the manufacturer, the manufacturer published reportable range must be used.
g. If the reportable range study indicates a usable range smaller than the limits indicated by the manufacturer, the smaller range must be used.

4. Reference Range Verification.

Reference ranges are a measured set of values determined to occur in a healthy non-diseased population. The laboratory must verify that their choice of Reference Range is valid for their study population.

The choice of Reference Range can be from documented literature, manufacturer suggested ranges, existing laboratory range, or the lab may perform a full normal value study to evaluate their own range.
If a lab decides to use published ranges, these ranges must be verified. To validate or transfer of this published range, the lab must analyze specimens from 20 healthy, non-diseased individuals, for each subgroup. If 2 or fewer results fall outside the published range, it is considered validated. If, however, more than 2 results fall outside the published range, a more extensive study should be conducted.

The Laboratory Director is the ultimate decision maker on which validation to use based on the study population.

5. Carry Over Studies

Demonstration of assay carry over is also required for all HPTN study related procedures.

Any deviation from the manufacturer's recommendations will put that procedure into the modified category.

Studies for quantitative assays that are not FDA approved, or are FDA approved and have been modified must also contain all of the previous items 1-5as well as the following:

6. Analytical Sensitivity:

This is the lowest measurable concentration that is distinguishable from zero.

Successive dilutions of a previously analyzed patient specimen or control can be used.

7. Analytical Specificity:

This is the ability to deal with interfering substances.

At a minimum, run samples spiked with hemoglobin, bilirubin and lipids.

8. Any other applicable performance characteristics:

Demonstration of carryover is one example.

ACCEPTABILITY CRITERIA

The Laboratory Director must set the limits for assay acceptability.

In the absence of a Laboratory Director, a designated responsible individual from the site can set the criteria. HPTN Central Laboratory staff may be able to offer guidance for setting limits.

Method Validation		
SOP Number:	Total Number of Pages:	
Primary Author: E. Livant	Signature & Date:	
Reviewed by:	Signature & Date:	
Revision History:		
Distributed to:		
Approved by:	Signature & Date:	
Distribution Date:	Effective Date:	Revision Due Date:

PURPOSE

When new instruments or test methods are introduced into the laboratory it is essential to evaluate their performance in the location and conditions under which they will be used and to verify specifications provided by the manufacturer. This process is known as *Method Validation.* This document provides explanation and basic guidelines for performing method validation; basic information regarding the method validation process. More detailed information can be found in publications from the Westgard Quality Corporation, the National Committee on Clinical Laboratory Standards (NCCLS) and many other sources.

SCOPE

This SOP applies to all method validations performed in the YRG CARE Infectious Diseases Laboratory. The application of these validations will be carried out at the discretion of the Laboratory Manager based on available resources, staffing and funds.

Summary reports must be kept on file of all method validation procedures performed. These reports must be signed and dated by a laboratory manager, division in charge or designee.

This document does not cover validation of Laboratory Information Systems or other computerized informatics systems. When these types of systems dealing with laboratory

results and/or patient information are implemented in the laboratory, a plan should be devised to verify their performance against hard copy results.

OVERVIEW

1. Quantitative Test Methods
    a. Precision (Repeatability)
    b. Accuracy (Correlation)
    c. Linearity
        i. Analytical Measurement Range (AMR)
        ii. Clinically Reportable Range (CRR)
    d. Reference Range Studies
2. Qualitative Test Methods
    a. Individual Test Methods versus Testing Algorithms
    b. Sensitivity
    c. Specificity
    d. Predicted Value Positive (PVP)
    e. Predicted Value Negative (PVN)
3. Additional Testing Issues
    a. Lower Detection Limit (Analytical sensitivity)
    b. Analytical Interference (Analytical specificity)
    c. Specimen stability
    d. Deviations from Manufacturer's Recommendations

REQUIREMENTS FOR US GOVERNMENT SPONSORED STUDIES

Laboratories which perform testing for United States Government funded studies (NIH, FDA, CDC, HPTN, ACTG, DAIDS etc…) need to be in compliance with any project specific regulations and The US Clinical Laboratory Improvement Act (CLIA) of 1987:

- For *unmodified* FDA approved quantitative methods, the site must locally validate accuracy, precision, linearity and reference ranges.

- For *modified* FDA approved or non-FDA approved quantitative methods, the site must locally validate accuracy, precision, linearity, analytical sensitivity, analytical specificity and reference ranges.
- For qualitative methods, the requirements are based on the direction from the sponsor of the study.

LABORATORY TEST METHOD VALIDATION TERMINOLOGY

Accuracy: How close test results come to the actual value.

Clinically Reportable Range: The range of analyte values that can be reported to the clinician for a quantitative result after appropriate specimen dilutions or concentrations; an extension of the AMR (See linearity, below).

Coefficient of Variation: a statistical measure of the dispersion of data points around the mean in context of the magnitude of the mean

Correlation: a statistical comparison between two instruments or test methods

Data Cleaning: the process of checking data sets for errors and putting all data in appropriate standard formats to facilitate analysis

Gold Standard: (*reference method*) an instrument or test method that is designated as the most reliable or accurate available for a given situation

Linearity: (*Analytical Measurement Range (AMR)*) assessment of the lowest and highest level at which an analyte can be accurately measured without any type of specimen dilution or concentration that is not part of the standard assay procedure

Mean: the average of a set numbers

Normal Distribution: (*Gaussian distribution*) a distribution of data that shows characteristics of the traditional "bell curve". The distribution has the most data points at the mean with the number of data points decreasing the further away from the mean. The curve, when divided at the mean, is a mirror image of itself. The normal curve has the characteristic that approximately 68.2% of the values are located ± 1 standard deviation from the mean, 95.5% of the values are located ± 2 standard deviations from the mean and 99.7% of the values are located ± 3 standard deviations from the mean.

Parametrics: Statistical tests which may be used when certain criteria are met such as data with a normal distribution. When these criteria are not met, weaker statistical tests (non parametrics) must be used.

Precision: The degree of reproducibility among several independent measurements of the same sample (or sample pool) for the same analyte

Predicted Value Negative: given a negative screening test result, the probability of a negative gold standard result

Predicted Value Positive: given a positive screening test result, the probability of a positive gold standard result

Qualitative test: A test method which gives a result that is categorical; frequently positive or negative

Quantitative test: A test method that gives a result which is a number with specific units

Random Error: error which may be positive or negative, with no predictability in terms of direction or magnitude

Reference range: (*normal range, reference interval*) for each quantitative analyte, a statistically generated span which is calculated from a study of healthy individuals to be used for interpretation of results.

Regression analysis: A statistical technique applied to data to determine, for predictive purposes, the degree of correlation of a dependent variable with one or more independent variables

Screening test: a simple, rapid, easy test method; used to test large numbers of samples; frequently will need to have results verified by a second test method

Sensitivity: given a positive gold standard result, the probability of a positive screening test result

Specificity: given a negative gold standard result, the probability of a negative screening test result

Standard Deviation: a statistical measure of the amount of dispersion of data points around a the mean

Systematic Error: error which is mostly in one direction, may be of consistent magnitude and can be predicated

Testing algorithm: a predefined process of multiple lab tests, used to determine one final lab result.

INSTRUMENT/ TEST SELECTION

It is essential to select the appropriate instrument or test method. Selection criteria will differ between laboratories. A complete discussion of instrument selection is beyond the scope of this document but a partial list of selection criteria includes:

- ✓ Your intended use for the instrument or test (Clinical, research or both)
- ✓ Anticipated testing volume
- ✓ Instrument cost
- ✓ Reagent/Kit
    - o Cost per test
    - o Storage requirement (Bulk, temperature)
    - o Dry versus liquid reagents
    - o Shelf life
- ✓ Test Menu
- ✓ Vendor service
    - o Installation and training
    - o Service contracts
    - o Technical/ troubleshooting: online, telephone
    - o Responsiveness to questions and problems
- ✓ Physical Specifications (size, weight) of the instrument; available space in the laboratory
- ✓ Complexity of usage, staffing requirements
- ✓ Throughput (tests per hour, duration of assay procedure)
- ✓ Available controls and calibrators
- ✓ Methodology
- ✓ On board Informatics specifications
- ✓ Electrical requirements
- ✓ Other equipments needed to use instrument
- ✓ Are there other similar instruments in your area? (What is their experience?)
- ✓ Does the instrument manufacturer also make reagents? (Preferable)
- ✓ Published instrument/ test performance (Literature or kit inserts)
- ✓ US FDA approval (relevant mostly if conducting studies for American interests)

DATA AND REPORTING CONSIDERATIONS

Pre testing preparation

Before starting validation of an instrument or test method the following steps should be completed:

- ✓ The installation of the instrument should be completed satisfactorily
- ✓ Staff should have received enough training to perform the testing correctly
- ✓ Appropriate reagents, controls, calibrators, test kits and supplies should be acquired and onsite in sufficient quantity to complete the validation procedures
- ✓ A basic plan specific to the validation of the particular instrument or test method should be drafted
    - o Timetable
    - o Responsibilities
    - o Source of specimens to be used
        - ▪ Ethics approvals as relevant if human specimens are to be used or need to be acquired
        - ▪ Acceptability criteria for specimens
    - o Analysis plan
    - o Publication potential (sometimes applicable for reference range studies and instrument comparisons)

Testing

All testing must be performed by authorized personnel using equipment, supplies, reagents and procedures as those used for testing of patient specimens for clinical and research lab results reporting (Good Laboratory Practices). Quality Control (QC) must be performed for all tests per established protocols before performing testing for method validation.

Source Documents

Source documents must be defined for each new test method before testing begins. For method validations, source documents must be maintained for the life of the instrument.

Source documents will follow the current YRG CARE guidelines and standards (refer to the current YRG CARE Quality Assurance SOP). These records must clearly show who performed testing on what date, results of corresponding QC, and reagent lots with expiration dates.

Data Entry

It will usually be advantageous to have the data from validation experiments transferred from instrument electronic copy or hard copy to electronic spread sheet for analysis (data entry). This is a crucial step which requires extreme diligence to avoid errors which will lead to false conclusions. It is recommended that data entry be checked by a second person against source documents (QC and "data cleaning"). For larger data sets, double data entry data bases may be beneficial as they can give 100% review of results entry; consult advice from IT specialists.

Analysis

Again, it is recommended that data is entered into a program with statistical capabilities for analysis purposes. The choice of program is at the discretion of the person performing the analysis. Statistical programs include but are not limited to Excel, SPSS, SAS, STATA and EPI INFO.

Simple statistical analysis can frequently be performed by laboratory staff. More advanced experience is advised for more advanced methods. Data should not be simply plugged into statistical programs without an understanding of the statistical assumptions involved.

Data should be removed from analysis if there were obvious errors in testing or with the specimen. In certain cases, outliers produced under normal testing conditions may be removed from analysis. This must be done with care because this can mask true problems with instrument performance. Any time outliers are removed from analysis, it must be noted in the final validation report and the removed data must be presented in a separate table.

Interpretation

Whenever possible, it is advisable to have preset acceptability limits determined. In many cases however, it will be necessary to use judgment to determine if the instrument or method has been successfully validated. Included in the final report must be this final conclusion of the validation testing; rationale for the conclusion should be elucidated when it is not obvious. If the validation is not completely successful further testing or other options for corrective action should be recommended.

QUANTITATIVE TEST METHODS: PRECISION

This is a replication experiment to measure random error. The same sample material is tested at least 20 times and these results are used to calculate mean, standard deviation and coefficient of variation. 20 is the minimum but it is advisable to perform more when possible; after initial validation , precision can be rechecked on larger sample sizes from QC runs of the same lot.

Experiment duration

The precision experiment can be done

- "Intra or within" run: in this scenario all of the runs are done at once. This should have the least amount of variation and should yield the best results.
- "Inter or between" run: in this case the testing will take place over several different runs back to back on the same day. This will have more inherent variation and is a more stringent test.
- Between day: testing is performed over a period of days. This allows for the most variation and is the most stringent. This experiment most closely mirrors real life use of the instrument.

It is best to have between day precision data. If the results from between day data are deemed unacceptable, perform inter run and intra run experiments. Based on these results, identify sources of imprecision (examples may include reagent stability, sample stability, different technique between operators, environmental conditions, etc…), take corrective action and repeat the experiment.

Sample

Controls or calibrators are best to use, especially when experiments will take place over a period of several days as they generally have longer stabilities. Human samples may be used when a sufficient amount is available but should not be used for long periods of times if they may deteriorate and yield falsely imprecise results.

By the end of validation testing, precision should have been tested at least at the same number of levels as controls that will be run for the instrument. If samples other than controls are used, they should cover approximately the same range as the controls. Precision must be tested at a minimum of two levels.

Interpretation

See Appendix B for calculations for mean, standard deviation (SD) and coefficient of variation (CV). There is no single set level of acceptable precision that can be applied to all analytes in every clinical laboratory. Available commercial methods vary in precision for different test methods. Laboratories should decide acceptable levels of precision based on the needs of the facility. The locally generated statistics should be comparable to those provided by the manufacturer in most cases.

The CV and SD need to be both considered when evaluating results from a precision study. If the sample being tested has a very low mean (with regard to the manufacturer's reference range or linearity) then the CV may be falsely elevated. If the standard deviation is low, then it may acceptable. Conversely, if the mean is very high, the CV may be falsely lowered.

When using control materials for precision runs, the results do not have to be within manufacturer's ranges. The lab will establish its own ranges after completion of validation testing. Results that are grossly outside of manufacturer's ranges should alert the user to a potential problem with instrument and troubleshooting should take place before proceeding.

QUANTITATIVE TEST METHODS: CORRELATION

This experiment tests 30-40 random samples in order to assess systematic error and accuracy. (40 is the US industry standard minimum. Some sources recommend using as many as 100 samples. These decisions must be based on availability of samples and funding.) The samples are tested in duplicate on two of the same instrument or two different instruments. In ideal situations, one third of the samples should be from the low range of the instrument linearity, one third should be from the high range of the instrument linearity, and one third should be from the middle range of the instrument linearity.

Choice of comparison instrument

If a gold standard instrument is available this is ideal. Otherwise it is advisable to find a reliable instrument of the same type or methodology as the one to be validated. If different methods are used it may be difficult to asses which instrument is in error when differences are found. If the reference instrument is based in another laboratory, every effort should be made to assess that laboratory's compliance with Good Laboratory practices. If no reliable comparison instrument exists, the experiment should not be performed.

Sample testing

Random human samples are preferable since a wide range of results is needed. Attempt to find samples which cover the manufacturer's linearity of the instrument. The samples should still be viable for testing for the analytes in question.

The samples should be tested on both analyzers at approximately the same time to reduce false error cause by sample degradation. If the two instruments are not located at the same facility, care must be taken when transporting the samples to avoid sample degradation.

Interpretation

The analysis may be done using different statistical correlation models with the software of choice. Linear regression is frequently used. Statistical tests (such as T tests) may also be used to compare the means. An X-Y graph should be prepared with a plot of the best fit line. A difference plot may also be generated. A detailed explanation of these methods is beyond the scope of this document. Proper training in statistics and computer programs is required.

For regression, one will want to generate three basic statistics: correlation coefficient (frequently R or r), slope (of regression best fit line), and Y-intercept (of regression best fit line). The correlation coefficient is a measure of how well the two methods agree. The slope is the angle of the line. The Y-intercept is where the line crosses the Y axis. The ideal correlation coefficient and slope is one. The ideal Y-intercept is 0. See Appendix C for an example of an X-Y graph with these conditions-a perfect correlation. In this situation both methods achieved exactly the same result for each sample tested. It will be rare in actual practice to achieve a perfect correlation, so some judgment will be needed when evaluating results. When possible, define acceptable limits of error before proceeding.

Some cases may have a slope close to 1 with an intercept not close to zero In these cases one of the methods is systematically giving measurements at a higher level than the other, but that this difference is a consistent amount through the range of possible values. In certain cases this may indicate that the new instrument should be recalibrated and the experiment repeated. In certain cases this may be an acceptable situation and the difference in measurement will need to be reflected in reference ranges for both instruments (see below).

If the slope is far from 1, this indicates that one method is systematically giving measurements at a higher level than the other, but that this difference may not be consistent through the range of possible values or may be a consistent percentage

increase. This may require recalibration or other corrective action but these two methods are generally not compatible unless the problem can be corrected.

A correlation coefficient that is low indicates a weak relationship between the two methods. This may be caused by an unacceptable level of random error and should be corrected before the new method is put into use.

QUANTITATIVE TEST METHODS: LINEARITY

This experiment involves taking a sample with a known high concentration and making 5-6 dilutions with appropriate diluent. These dilutions are then tested and these results are compared to the expected values (based on dilution factors). The results are evaluated by plotting the results on an X-Y graph and determining the linearity by visual fit of a manually drawn line (do not use a statistical program to fit the line).

The linearity or analytical measurement range (AMR) of an instrument is the range where it can accurately measure analytes as evidenced by the straightness of the line plotted. The AMR of the test method is range of measurements that can be determined to be accurate without any type of dilution or concentration procedures.

From the AMR, the Clinically Reportable Range (CRR) will be determined based on manufacturer's recommendations and in consultation with medical staff. Samples that are above AMR may be diluted to obtain a result within the AMR; this result is then multiplied by the dilution factor to calculate the actual result. The dilution factor should not exceed the manufacturer's recommendations. Thus based on the dilution factor limit and clinical significance of the test, the CRR will be established.

Samples which give results below this CRR must be reported as "less than" the lower CRR limit after confirmation (more testing must be performed for non FDA approved methods to verify the lower detection limit-see below). Samples which give results above the CRR must be reported as "greater than" the upper limit of the CRR.

Examples: For test A, the AMR (Linearity) is 5-600 mg/dL. The CRR is 10 to 1000.

- Sample 1 gives an initial result of 700. The sample is diluted 1:2 which gives a result of 375. The result is reported to the clinic as 750.
- Sample 2 gives an initial result of 3 and verified by repeat testing. The result is reported to the clinic as <10.
- Sample 3 gives an initial result of 1200. The sample is diluted 1:3, retested and has a result of 1150 after multiplication by the dilution factor. The result is reported to the clinic as > 1000.

Sample

Controls or calibrators may be used, assuming they are available with a known high value. A patient specimen may be used if found to have a high value which is confirmed by a second method. The diluent used must be approved for the type of sample chosen. The College of American Pathologists (CAP) produces linearity panels for many methods. These may be used for validation of methods but the validated range will only be between the high and low levels provided in the CAP panel.

QUANTITATIVE TEST METHODS: REFERENCE RANGE

All quantitative results must be accompanied by a reference or normal range. These ranges are known to differ in different regions because of environmental, socioeconomic and genetic factors. Different test methods or instruments may also cause differences. Thus, the reference range must be established locally for each different instrument and test method. Reference range studies must be repeated when new instrumentation is purchased or at least every 5 years.

Study participants

A study sample that is somewhat representative of the normal, healthy client population for the laboratory will tested to establish the normal range. The first steps are therefore to characterize the client population for the laboratory and to establish criteria for what constitutes a “normal” or “healthy” person. Sub groups within the client population (gender, pediatric, geriatric) must be identified. The sample sizes will range from 20 to 120 for each sub group analyzed (See analysis section)

Screening

A screening process must be developed to evaluate willing volunteers for eligibility. Inclusion and exclusion criteria must be established to select normal volunteers. The screening process usually involves a health questionnaire and may involve physical examination. Special attention must be paid to the reproductive status of women. Informed consent must be administered based on local governmental and institutional regulations. When resources are available, other testing may be performed such as chest X-rays and electro cardio grams. Once participants have been deemed eligible by non invasive methods, blood samples may be taken. Further testing may take place on these samples to determine if sub-clinical conditions (example: HIV seropositivity) exist which should preclude them from inclusion; the decision to conduct this testing is based on local prevalence and clinical significance. Women should be evaluated for pregnancy and menstrual status.

Testing

The testing should be performed by standard methods as would occur with normal patient testing for all analytes in question.

Analysis

After 20 valid samples are tested for each subgroup, these results may first be examined. If 19 or greater of the results for a given analyte are within the manufacturer's range, then this range may be considered validated for the local population. If 18 or fewer results for a given analyte are outside the manufacturer's range, then it is not validated.

In this case, 120 samples must be analyzed for each subgroup. Outliers may be removed here based on judgment or by use of statistical tests; when these outliers are removed they will need to be replaced by additional values to achieve the required sample size. Parametric or non parametric methods will be employed based on the normalcy of the sample distribution to establish the mean and 95% of the distribution. When a normal range has an upper and lower limit, the central 95% of the distribution is calculated. When the normal range is presented as "less than" or "greater than", the upper or lower

95% will be calculated. One may also wish to calculate confidence intervals for the reference ranges. Consultation with a statistician is recommended.

See Appendix D for a formula for evaluating outliers and non parametric establishment of 95% of the distribution.

QUANTITATIVE TEST METHODS: ANALYTICAL SENSITIVITY AND SPECIFICITY

The steps involved in these methods will not be covered in detail in this document because YRG CARE laboratory will generally be using FDA approved methods and these experiments only need to be performed as desired by the laboratory. They are described in detail in the Westgard publication "Basic Method validation."

If the laboratory undertakes certain specific types of testing where extremely low levels of detection are important (forensic drug testing, certain tumor markers, some hormone levels), it may be necessary to perform additional experiments to verify lower detection limits (analytical sensitivity). Consultation with outside expertise is recommended in these cases.

QUALITATIVE TEST METHODS

A laboratory may wish to evaluate performance of tests which do not yield an actual number as the final result. This is usually done by comparison to an existing gold standard method or another established method. Tests may be evaluated individually or testing algorithms may be examined.

100 positive (by gold standard) and 100 negative (by gold standard) samples should be tested when comparing 2 different methods. If two of the same instrument are being compared to validate a new purchase, 20 positives and 20 negatives may be tested. Patient samples should be used when available. After testing by both methods and instruments, the results should be classified into one of for cells based on positive/negative results for each method (see Appendix B.4).

Sensitivity, Specificity, Predicted Value Positive and Predicted Value Negative can then be calculated from the totals in each cell. The acceptability of these results will depend on the test being evaluated. Cost, gravity of test result, difficulty of test and application of the test must all be considered.

GENERAL TESTING ISSUES: SPECIMEN STABILITY

Manufacturers of tests should provide information regarding specimen storage requirements and stability. In certain instances this information is not available. In these cases, the laboratory should perform local validation to determine the storage and stability criteria for specimens.

Specimens of the appropriate type are obtained from patients and tested immediately. They are then stored in specific conditions and retested at intervals until the results are deemed unacceptable. This information can be used to determine the local criteria for specimen storage.

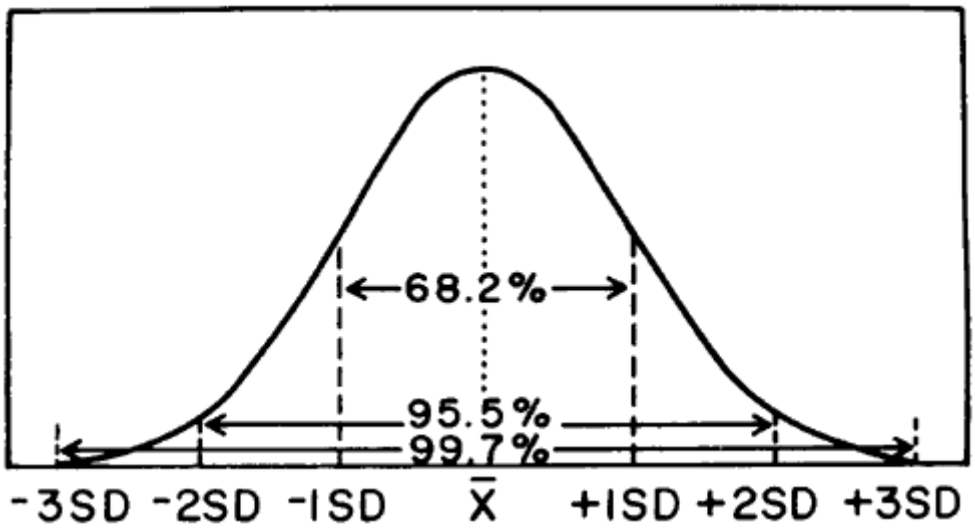
For quantitative tests, a test may be considered "unacceptable" when the results have changed clinically significantly from the original result. For qualitative tests, when the result is no longer the same as the original or when interpretation of the test becomes difficult, it can be considered unacceptable.

GENERAL TESTING ISSUES: DEVIATIONS FROM MANUFACTURER'S RECOMMENDATIONS

It is generally discouraged to use commercial tests and instruments in a fashion not in accordance with manufacturer's recommendations. Laboratories may need to do this in certain instances. Testing should be undertaken to demonstrate that these deviations will not affect the quality of results. The nature of this testing would depend on the type of deviation; laboratories will need to design individual experiments accordingly. Examples of deviations include (but is not limited to), changing of specimen type or storage conditions, extension of specimen stability, altering packaging of reagents or changing of

incubation times. Note that these modifications may also require that the lab validate the analytical sensitivity and specificity of the test.

Appendix A: The normal curve



Where
$\overline{X}$ = the mean
SD = the standard deviation

Appendix B: Statistical formulae

1. Mean

$$\overline{X} = (x_1 + \ldots\ldots + X_n) / n$$

Where
$\overline{X}$ = the mean
n = the number of measurements
$(X_1 + \ldots\ldots + X_n)$ = the sum of all the measurements, X_1 being the first and X_n being the last.

2. Standard Deviation

$$s = \sqrt{\frac{\sum(x_i - \overline{x})^2}{(n - 1)}}$$

Where
S (or SD) = the standard deviation

Σ = the sum of whatever follows it
$\overline{x}$ = the mean
x_i = each value
n = the number of values

3. Coefficient of Variation

CV = (SD/$\overline{x}$)*100

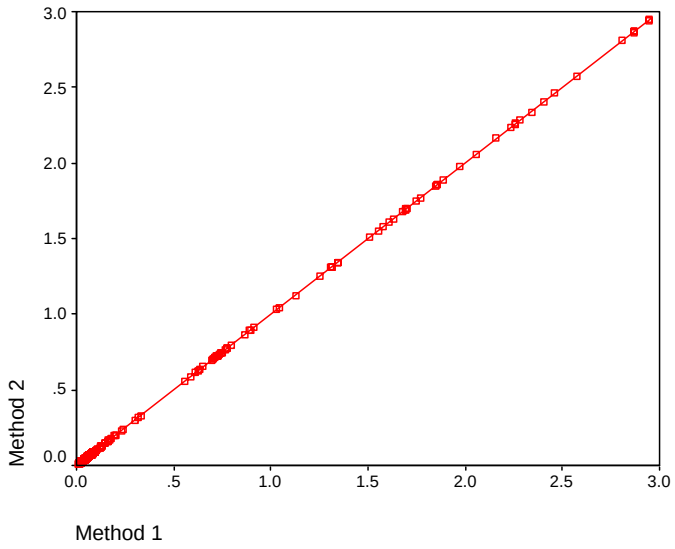
Where
CV = Coefficient of Variation
SD = the standard deviation
$\overline{x}$ = the mean

4. Qualitative Test Statistics

	Gold Standard Positive Results	Gold Standard Negative Results
Screening Test Positive Results	**A**	**B**
Screening Test Negative Results	**C**	**D**

Sensitivity: A/ (A+C)
Specificity: D/ (B+D)
Predicted Value Positive: A/ (A+B)
Predicted Value Negative: D (C+D)

Appendix C: Perfect Correlation Graph



Appendix D: Reference Range Calculations

Outlier Calculation

1. R: Range from lowest observation to the highest observation including extreme value

2. D: The absolute difference between the most extreme observation (high or low) and the next most extreme observation

3. Outlier removal rule: if D/R > 1/3 then outlier is removed

4. Application of rule: when an outlier is removed, the process should be reapplied to the observation which is now the most extreme. When 2 or 3 outliers exist on one side of the distribution, this calculation will not be effective and judgment should be employed to remove outliers. The data should always be plotted to help evaluation.

Non Parametric Determination of Boundaries

1. Let n= the number of observations.

2. Arrange the observations in order of magnitude from low to high.

3. Assign each observation a rank, starting with one for the lowest.

Double Boundary

Calculation of the lower 2.5 percentile and the upper 97.5 percentile to estimate the central 95%:

1. The lower limit is calculated by the formula 0.025 (n+1). This value is rounded to the nearest whole number. This number corresponds to the rank of the observation which will be used for the lower limit of the normal range.

2. The upper limit is calculated by the formula 0.975 (n+1). This value is rounded to the nearest whole number. This number corresponds to the rank of the observation which will be used as the upper limit of the normal range.

Single Boundary

Calculation of the lower 95 %:

The lower 95% is calculated using the formula 0.95 (n+1). This value is rounded to the nearest whole number. This number corresponds to the rank of the observation which will be used for the lower limit.

REFERENCES

1.Westgard JO, Basic Method Validation; 1999; Westgard Quality Corporation

2. NCCLS C28-1: How to define and determine reference range intervals in the clinical Laboratory- Second Edition- Approved Guideline Villanova, PA: National Committee for Laboratory Standards, 2000

Validation Protocol Form

Purpose: ☐Validation ☐Re-Validation ✕Other:
Version #/Software Upgrade #:_____N/A_____

Description of Equipment/Process:
Equipment/Process:
Serial Number
Location:
Other: N/A

Procedure:
Refer to SOP #:

Results: (Attach additional pages with results if needed)

Conclusions:

Acceptable:
Pass/Fail: N/A
Other: N/A
Expected Ranges: N/A

Recommendations/Corrective Action:

Validation Performed by: _____N/A_____

Date of Validation: ____N/A_____

Designee review/Date: _____

Section 6: Personnel

Qualified and properly trained staff is essential for the performance of any laboratory. All HPTN/MTN laboratories must follow steps to ensure that have a well a quality workforce. These guidelines also apply for non-lab staff who perform laboratory procedures (such as nurses performing rapid tests in clinics) and laboratory staff that only perform a test on a rare basis.

Employees must have education and experience appropriate for the positions they hold. Each technical employee must have on file diplomas and education records, training certificates, CV or résumé, and any relevant licenses or professional memberships. Each employee needs a job description; this should be signed by both the employee and his/her supervisor.

Before beginning to perform lab tests or other activities, employees must be properly trained. This training must be documented for any test or other major lab activity such as phlebotomy. In cases where these guidelines are being implemented in existing labs where training was not documented, the lab should make summaries of past training as well as possible.

Competency can be assessed through many methods including:

- direct observation of employee by a supervisor or senior technician
- employee testing of unknown specimens
- written or oral exam
- performance of proficiency testing
- proper handling of difficult specimen
- proper handling of instrument malfunction.
- ability in handling trouble shooting and problem solving skills.
- safe handling of instruments and infectious specimens

Competency must before the employee begins performing the test or activity, after six months, twelve months and each year after that. If there is any indication that the employee is lacking competency, retraining should occur and competency be reassessed.

Documentation should be maintained for training and seminars attended by the staff member. Continuing education opportunities should be offered to staff so that they can further their careers and maintain there knowledge of the field.

KILIMANJARO REPRODUCTIVE HEALTH PROGRAM MOSHI, TANZANIA

MAWENZI CLINIC LABORATORY

EMPLOYEE TRAINING CHECKLIST
BECTON DICKSON PROBETEC

Employee Name: _____

Date of Hire: _____

Procedure	Employee Signature	Date	Training Staff Signature	Date
Specimen Processing				
Software Use				
Test Procedure				
Interpretation of Results				
Quality Control				
Clean-up				
Maintenance				

Supervisor Review: _____

KILIMANJARO REPRODUCTIVE HEALTH PROGRAMME

CLINICAL LABORATORY EMPLOYEE COMPETENCY PROGRAM

TEST PROCEDURE: Focus HSV-2

EMPLOYEE:

DATE OF COMPETENCY TESTING:

Specimen Identification #	Employee Result	Known Value	Result Acceptable?
			Yes No
			Yes No
			Yes No
			Yes No
			Yes No
			Yes No

QC Results:

Other Competency verification method (Describe):

Employee results acceptable? Yes No

Comments:

If no, corrective action recommended:

Approved by: Date

Section 7: Facilities, Reagents and Equipment

Laboratories need to have adequate physical space to perform testing and provide a safe work environment. The space must be properly lit and ventilated. Temperature and humidity should be controlled for the comfort of the staff and for preservation of equipment, reagents, and specimens. All work areas should be kept clean and organized.

Specific attention must be paid to power supply; equipment purchased from the United States will use 110 volts while equipment from most other countries will use 220 volts. All equipment should be checked prior to use to ensure that the equipment functions properly and is not damaged. Voltage converters may be used but there should be adequate labeling in these cases to prevent people from plugging a piece of 110 volt equipment directly into 220 volt sockets. Voltage stabilizers and uninterrupted power supply units are crucial in areas where power fluctuations and outages occur. Many facilities will need an automatic back-up generator.

All equipment must have a preventative maintenance plan based on the manufacturer's guidelines or other industry references. All maintenance, planned or unplanned, must be documented. It is advisable to purchase service contracts for major pieces of equipment such as analyzers. Any problems encountered with equipment must be documented with the type of problem, date and time it occurred, and follow up actions. These logs should be available to staff using the equipment as a reference of past problems to aid in troubleshooting.

Temperature sensitive equipment needs to have a schedule of temperature checks. For refrigerators and freezers where specimens or reagents are stored, they must be checked 7 days a week, 365 days a year and generally twice a day. When temperatures are out of range, there must be documentation of corrective action and the condition of the specimens or reagents involved. All labs must have access to a National Institute of Standards and Technology (NIST) thermometer or equivalent to verify all thermometers in use twice a year.

All pipettes and balances must have their performance verified twice a year. Centrifuges must have their speed checked by tachometer twice a year. Timers need to be checked twice a year. Biological safety cabinets need to be certified by a qualified technician once per year.

All equipment should be given a facility ID number and tracked in an inventory including the location, serial number, make and model, and other relevant information.

Proper handling and inventory of reagents is crucial. All reagents must be properly labeled including contents, receive date, open date and initials of who opened them as well as preparation date and expiration date.. Reagents must be closely monitored to ensure they have not expired. All reagents must be stored and transported per manufacturer's instructions. Refrigerators, freezers and room temperature ranges need to take into account the reagents stored and the most stringent range used.

The use of expired reagents is never encouraged and in most cases is prohibited; CAP accredited laboratories may never use expired reagents. In countries where shipping and customs may be problematic, extra attention needs to be focused on inventory of reagents to assure adequate

supplies. A sample SOP is included which addresses expired reagent use. It is recommended that this issue is discussed with the HPTN and/or MTN NL's if it is a potential issue.

If the laboratory has a water purification system, the quality of the water must be checked on a regular basis i.e. at least each working day. This must be documented on a chart which may include a comments/corrective action section or corrective action may be recorded on another form.

The following procedures and specifications are for the testing of water which has been purified for clinical laboratory use. There are three grades of water recognized along with the minimum specifications for bacterial count for each.

Type I	Used for the preparations of solutions, reagents (EIA testing) requiring minimum interference and maximum precision and accuracy (10cfu/ml)
Type II	Used for general laboratory testing other than described above
Type III	Used for glassware washing, but not final rinsing and for feedwater for production of higher-grade water

The preferred water is Type I, distilled, deionized water. If this is not available, distilled water can be used and sterilized if necessary.

SOP Number: TBD **Title:** Ambient Temperature and Equipment Temperature Monitoring

Original Effective Date: Not Applicable **Revision Effective Date:** Not Applicable

Author: Grace Mhango, Kilimanjaro Reporductive Health Project

1. Purpose

The proper storage of specimens and test materials, as well as the processing of the specimens under ideal temperature conditions are factors that contribute towards assuring the quality of results produced in the laboratory. It is therefore important that all laboratory staff understand their responsibility with respect to the close monitoring of temperatures of the laboratory working spaces and of temperature-sensitive laboratory equipment.

2. Scope

This procedure applies to all temperature-sensitive equipment in the laboratory, as well as all areas in the laboratory in which specimens are processed and non-refrigerated reagents or test kits are stored.

3. Responsibility

All KRHP laboratory personnel: monitoring of temperatures, corrective action, reporting of problems to supervisors and performance of thermometer checks and calibrations when needed.

Laboratory manager or designee: monthly review of temperature charts, maintenance of records, corrective actions in situations where lab staff can not correct temperature irregularities.

4. Temperature Monitoring

The ambient temperature of all lab areas where specimens are processed or non-refrigerated reagents stored, as well as the temperatures of all temperature-sensitive equipment shall be monitored twice daily during weekdays and once a day on Saturdays.

During weekdays and working Saturdays, temperatures will be checked and recorded in the morning between 7:00 a.m. and 9:00 a.m., and in the evening between 5:00 p.m. and 7:00 p.m., unless otherwise indicated. On non working Saturdays, Sundays and Holidays the temperature will be recorded between 9:00 a.m. and 12:00 p.m.

All equipment and ambient temperatures will be recorded manually on charts bearing the following information:-

- Equipment identification number (where applicable)
- Equipment make, model and capacity (where applicable)
- Location of equipment in lab, or room where ambient temperature is to be monitored
- Required temperature
- Date (the dates for the entire month are pre-printed on the chart)
- Time (morning and evening)

- Temperature (morning and evening)
- Initials (morning and evening)
- Comments (for corrective actions, equipment servicing details, etc.)

The charts will be changed on the last day of each month, after the evening temperature reading has been taken.
Where Dickson chart recorders are used, the temperature will be plotted continuously on the Dickson charts provided. In addition, the temperature on the digital display of the Dickson thermometer will be recorded manually twice a day during week days and once a day on Saturdays, as described above. The charts used with the Dickson recorders will be changed every Monday morning.

4.1 Temperature Monitoring: Ambient Temperature

4.1.1 Materials and Equipment

4.1.1.1 VWR Traceable Monitoring Thermometer

4.1.1.2 Ambient Temperature Chart

4.1.2 Acceptable Temperature Range

The acceptable ambient temperature range of the lab working areas is **15°C** to **30°C**.

4.1.3 Procedure

4.1.3.1 Read the temperature on the digital display of the thermometer and record it on the ambient temperature chart for the date indicated. The information required is as follows:

a. Time – the time that the temperature is recorded will be entered in a 12-hour time format (e.g. 7:30 a.m.)
b. Temperature – to be entered as displayed, with the units indicated (e.g. 26.5°C)
c. Initials of recorder

4.1.3.2 If the temperature is observed to be outside the acceptable range, a note of this must be made in the "Comments" section of the temperature chart, together with a brief description of the corrective action taken in response to this observation. The staff responsible for the corrective action must write the time and his/her initials next to the description of the corrective action.

The air conditioner may be used to regulate the temperature of the room so that it is restored to within the acceptable range.

4.1.3.3 The calibration of the thermometer used to monitor the ambient temperature will be checked against an NIST-certified thermometer once a year, in order to determine the accuracy of the thermometer.

4.2 Temperature Monitoring: Refrigerators

4.2.1 Materials and Equipment

4.2.1.1 Dickson SL4/SK4 Chart Recorder (self-recording thermometer)

4.2.1.2 Refrigerator Temperature Chart

4.2.2 Acceptable Temperature Range

The acceptable refrigerator temperature range is **2°C** to **8°C**.

4.2.3 Procedure

4.2.3.1 Read the temperature on the digital display of the thermometer and record it on the refrigerator temperature chart for the date indicated.

a. Time – the time that the temperature is recorded will be entered in a 12-hour time format (e.g. 7:30 a.m.)
b. Temperature – to be entered as displayed, with the units indicated (e.g. 5.0°C)
c. Initials of recorder

4.2.3.2 If the refrigerator temperature is observed to be outside the acceptable range, a note of this must be made in the "Comments" section of the temperature chart. A brief description of the corrective action taken in response to the temperature deviation must also be documented on the chart. The staff responsible for the corrective action must write the time and his/her initials next to the description of the corrective action.

The temperature control settings may be adjusted as appropriate in order to restore the refrigerator temperature to within the acceptable range. (Refer to refrigerator instruction manual.) In such a case, the temperature must be checked after an hour in order to assess the effectiveness of the action taken. This temperature will be recorded in the "Comments" section of the chart, together with the time and initials of the recording staff.

In case of over-icing, the refrigerator temperature may be corrected by defrosting the freezer. In this case, the laboratory staff must ensure that the instructions in the respective refrigerator operating manual are followed exactly.

Persistent out-of-range temperatures – such as below-range temperatures that cannot be corrected by adjusting the refrigerator temperature control settings – and problems noted at the end of the work day must be reported immediately to the KCMC Engineering Department (by telephone). The laboratory staff on duty will then submit a Laboratory Equipment Work Request form to Engineering. The work request to Engineering must be accompanied by an instruction manual of the refrigerator to be serviced.

4.2.3.3. The Dickson chart recorder will be used for continuous monitoring of the temperature of the refrigerators during the period in which no staff are in the laboratory, for instance after normal working hours. The laboratory staff responsible for checking the temperature after such a period must review the chart and make a note of any irregularities observed on the Refrigerator Temperature Chart. The staff must alert the supervisor immediately thereafter.

4.2.3.4 The charts that are used with the Dickson chart recorder will be changed every Monday morning.

4.2.3.5 The calibration of the thermometer used to monitor the refrigerator temperature will be checked against an NIST-certified thermometer once a year, in order to determine the accuracy of the thermometer.

4.3 Temperature Monitoring: -20°C Freezer

4.3.1 Materials and Equipment

4.3.1.1 Dickson SL4/SK4 Chart Recorder (self-recording thermometer)

4.3.1.2 Freezer Temperature Chart

4.3.2 Acceptable Temperature Range

The acceptable temperature range for this type of freezer is **-15 to -25°C.**

4.3.3 Procedure

4.3.3.1 Read the temperature on the digital display of the thermometer and record it on the freezer temperature chart for the date indicated.

a. Time – the time that the temperature is recorded will be entered in a 12-hour time format (e.g. 7:30 a.m.)
b. Temperature – to be entered as displayed, with the units indicated (e.g. -19.5°C)
c. Initials of recorder

4.3.3.2 If the freezer temperature is observed to be outside the acceptable range, a note of this must be made in the "Comments" section of the temperature chart. A brief description of the corrective action taken in response to the temperature deviation must also be documented on the chart. The staff responsible for the corrective action must write the time and his/her initials next to the description of the corrective action.

The temperature control settings may be adjusted as appropriate in order to restore the refrigerator temperature to within the acceptable range. (Refer to refrigerator instruction manual.) In such a case, the temperature must be checked after an hour in order to assess the effectiveness of the action taken. This temperature will be recorded in the "Comments" section of the chart, together with the time and initials of the recording staff.

The KCMC Engineering department will be contacted in the case of persistent temperature deviations. The procedure to be followed in reporting problems to the engineering department is as described in section 4.2.3.2.

4.3.3.3. The Dickson chart recorder will be used for continuous monitoring of the temperature of the freezer(s) during the period in which no staff are in the laboratory, for instance after normal working hours. The laboratory staff responsible for checking the temperature after such a period must review the chart and make a note of any irregularities observed on the Freezer Temperature Chart. The staff must alert the supervisor immediately thereafter.

4.3.3.4 The charts that are used with the Dickson chart recorder will be changed every Monday morning.

4.3.3.5 The calibration of the thermometer used to monitor the freezer temperature will be checked against an NIST-certified thermometer once a year, in order to determine the accuracy of the thermometer.

4.4 Temperature Monitoring: -86°C Freezer

4.3.1 Materials and Equipment

4.3.1.1 Inbuilt Thermoforma freezer thermometer

4.3.1.2 Freezer Temperature Chart

4.3.2 Acceptable Temperature Range

The acceptable temperature range for this type of freezer is **-70 to - 86°C.**

4.3.3 Procedure

4.3.3.1 Read the temperature on the digital display screen located on the freezer door and record it on the freezer temperature chart for the date indicated.

a. Time – the time that the temperature is recorded will be entered in a 12 hour time format (e.g. 7:30 a.m.)
b. Temperature – to be entered as displayed, with the units indicated (e.g. -85°C)
c. Initials of recorder

4.3.3.2 If the freezer temperature is observed to be outside the acceptable range, a note of this must be made in the "Comments" section of the temperature chart.

A brief description of the corrective action taken in response to the temperature deviation must also be documented on the chart. The staff responsible for the corrective action must write the time and his/her initials next to the description of the corrective action.

In case the suspected cause of a temperature drop is the prolonged opening of the freezer door, the freezer door must be checked to ensure that it is tightly closed. The temperature will then be checked after an hour in order to assess the effectiveness of the action taken. This temperature will be recorded in the "Comments" section of the chart, together with the time and initials of the recording staff.

The KCMC Engineering department will be contacted in the case of persistent temperature deviations. The procedure to be followed in reporting problems to the engineering department is as described in section 4.2.3.2.

4.5 Temperature Monitoring: 37°C Microplate Incubator

4.5.1 Materials and Equipment

4.5.1.1 Temperature indicator strip on bottom inner surface of Boekel Microplate incubator.

4.5.1.2 Incubator Temperature Chart

4.5.2 Acceptable Temperature Range

The acceptable temperature range for this incubator is **(To be determined)**.

4.5.3 Procedure

4.5.3.1 Read the temperature on the on the temperature indicator strip and record it on the incubator temperature chart for the date indicated. The information required is as follows:-

a. Time – the time that the temperature is recorded will be entered in a 12-hour time format (e.g. 7:30 a.m.)
b. Temperature – to be entered as read on the strip, with the units indicated (e.g. 37°C)
c. Initials of recorder

4.5.3.2 The temperature of the microplate incubator will be monitored twice daily; once when the instrument has been switched on for incubation of the first ELISA run of the day, and also after the plate from the last ELISA run of the day has been removed from the incubator. Allow thirty (30) minutes for the temperature to stabilise after switching on the instrument and after removing an ELISA plate before checking and recording the temperature.

4.5.3.3 Any out-of-range temperatures must be noted in the "Comments" section of the chart, as must any corrective actions taken in response. The staff responsible for the corrective action must write the time and his/her initials next to the description of the corrective action.

The incubator temperature may be corrected by adjusting the temperature screwdriver slot located at the back of the unit. Refer to the operating instructions for this procedure.

4.5.3.2 The accuracy of the thermometer used to monitor the incubator temperature will be checked against an NIST-certified thermometer once a year.

4.6 Temperature Monitoring: Water Bath

Water bath not in use. Equipment temperature range and monitoring details to be confirmed.

5. Data Management

5. 1 Completed charts that are used for manual temperature monitoring will be filed in the Archived Temperature Charts file, which is kept in the main KRHP lab space.
5.2 Used Dickson chart Recorder charts will be stored in the Dickson charts box in the main KRHP lab space.
5.3 The charts are subject to review by a supervisor once a month.

CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date:15/March/2006 Author: J. Westerman, MT(ASCP)
Title: BD ProbeTec ET Maintenance		Revised Effective Date: N/A Author: N/A
Proc#: VI.2.4	File Name: VI.2.4 BD ProbeTec ET Maintenance	Page 146 of 538

I. PURPOSE/PRINCIPLE: To provide a procedure for the maintenance of the BDProbeTec ET.

II. MATERIALS AND EQUIPMENT:

10% JIK (Bleach)
Deionized Water
Paper Towels
Lysing heater thermometer
Priming heater thermometer
Warming heater thermometer
BacTac solution
Thermal test plate
Normalizer Set
Hex head screwdriver

III. SPECIMEN TYPE / STORAGE: N/A

IV. FORMS:

VI.2.4A BD ProbeTec ET Equipment Maintenance Log
VI.2.4B BD ProbeTec ET Monthly Maintenance Log
II.A Equipment Corrective Action Log

V. PROCEDURE:

A. Pre-Run Maintenance:

1. Dampen a paper towel with 10% bleach wipe down all surface tops. Dampen a 2nd paper towel with distilled water and wipe down all surface tops.
2. Check the printer's paper supply each day. If the paper supply is low or exhausted, replace the paper.
3. Before each run, check the thermometer reading of the lysing Heater.
   a. Remove the cover and allow 15 minutes for the temperature to stabilize before checking the thermometer.
   b. Write the temperature of the thermometer on **IV.2.4A BD ProbeTec ET Maintenance Log**.
   c. If the thermometer reading is within 112 ºC -116 ºC, the heater is operating within its specification.
4. Before each run, check the thermometer reading of the Priming/Warming Heater.
   a. For the Priming heater, if the thermometer reading is within 72.5 ºC ± 0.5 ºC, the heater is operating within its specification.
   b. For the Warming heater, if the thermometer reading is within 54.0º C ± 0.5 ºC, the heater is operating within its specification.
   c. Document the temperatures on **IV.2.4A BD ProbeTec ET Maintenance Log**.
   d. Check the instrument operating temperature. If the LCD temperature display is 47.5 ºC- 55 ºC, the instrument is operating within its specification.

B. Post Run Clean-up:

1. After each plate is tested, you should perform the following cleanup activities:
   a. Clean the testing plates with 10 % JIK. Saturate the entire plate.
   b. Rinse with deionized water.
   c. Allow plate to air dry. The plate MUST be completely dry before reuse.

C. End of day Clean-up:

1. Clean countertops:

a. Wipe all countertops with 10 % JIK.
b. Allow solution to remain on the surface at least two minutes.
c. Using a clean towel, wipe the cleaned surface with deionized water.

2. Clean Lysing rack and Lysing Heater Cover:
   a. Soak the lysing rack with 10% JIK.
   b. Rinse thoroughly with deionized water.
   c. Allow to air dry.
   d. Wipe the cover with 10% JIK followed by deionized water.
3. Clean the Lysing Heater and Priming and Warming Heaters:
   a. With towels dampened with 10% JIK wipe the heaters. Follow with towels dampened with deionized water. **DO NOT IMMERSE THESE ITEMS**.
4. Clean the instrument exterior with 10% JIK, then wipe with deionized water.
5. Pipettor
   a. With towel dampened with 10%JIK, wipe down the pipettes, then wipe with deionized water.
   b. Recharge the pipettor.

D. Monthly Maintenance

1. Clean the air filter:
   a. The air filter is located on the rear of the BD ProbeTec ET instrument. To remove the filter, lift it up slightly, then slide the filter leftward (when facing the rear of the instrument).
   b. Wash the filter in a laboratory disinfectant. Place it on a paper towel and dry it thoroughly (if you are going to reuse it immediately).
   c. Replace the filter by sliding it rightward in to the filter housing. Pivot the bottom in toward the instrument, and lower the filter into place.
   d. Document the maintenance on VI.2.4B BD ProbeTec ET Monthly Maintenance Log.
2. Check the instrument temperature:
   a. Press the "carrier out" soft key.
   b. The thermal test plate remains in the instrument between runs.
   c. When the "okay to open door" icon appears, open the door and immediately read and record the temperature on the test plate thermometer. Do not remove the plate – perform the reading while the plate is on the carrier.
   d. The reading must be 52 ºC +/- 0.8 ºC. If it is not contact Becton Dickinson. Document any problems on **II.A Equipment Corrective Action log**.

E. As Needed / Annual Maintenance

1. Replace the pipettor batteries:

a. Remove battery case by positioning your thumb over the thumb grips and bracing your fingers against the front of the keypad. Apply firm pressure to the panel with the thumb grips to release the latch.
b. Carefully slide the battery case our of the pipettor, allowing sufficient slack for the attached wires.
c. Remove batteries by pressing a pipette tip against either battery through the slot in the bottom of the battery case.
d. Insert new batteries by placing the positive end into the battery case first, then the negative end. Make sure that the batteries are in the correct orientation.
e. Carefully insert the battery case into the pipettor. The case clicks when the latch is securely attached to the handle. If the case does not close easily, make sure that the wires are not obstructing.
f. Allow the replacement batteries to charge for 12- 14 hours before using the pipettor.

2. Normalizer Replacement:
   a. Normalizers should be replaced before they expire.
      1) The instrument provides two error messages that occur prior to the normalizer expiration. One occurs 30 days prior to expiration (**E21**) and the other is 10 days prior to expiration (**E22**).
      2) If the **E23** message appears, the expiration date has passed and the normalizers must be replaced before new plates can be logged in, modified, or plate tests can be initiated.
   b. Replace Normalizer:
      1) From the Main Status screen, press the "carrier out" soft key to move the carrier out to the load instrument.
      2) Turn off instrument power.
      3) Press the door release button and open the door.
      4) With the hex head screwdriver, loosen the four hex socket-head screws.
      5) Remove the cover/foam assembly and the old normalizers. **Note:** the old normalizers may need to be separated from the foam by pulling gently on the edge of the normalizer.
      6) Insert the new normalizers with the label facing upward and the words "FRONT OF INSTRUMENT" toward the front of the instrument. MAKE SURE the normalizer disks are fully seated in the holes.
      7) Replace the cover/foam and tighten the four screws. Note: Do not overtighten the screws.
      8) Close the instrument door.
      9) Turn instrument power on. (There will be Error Code 13 due to power failure).
      10) From the Main Status screen, press the "configuration" soft key. Continue to press the key until the Normalizer Login screen appears. See users manual for picture of the icon.
      11) Scan or enter the new normalizer identification number.

12) Press "move to next field" soft key "➔I". Use the UP or DOWN arrow to enter the month, date, and year.
13) Press the "exit" soft key.

VI. RESULTS/REPORTING:

Equipment maintenance logs are reviewed monthly by the supervisor/designee.

VII. LAB REFERENCE RANGE: N/A

VIII. LIMITATIONS: N/A

IX. SPECIAL NOTES:

Document any instrument failures on **II.A Equipment Corrective Action log**.

X. REFERENCES:

BDProbeTec ET User's Training Manual. Becton, Dickinson and Company. November 1999.

CIDRZ – Central Laboratory
Lusaka, Zambia

BD ProbeTec ET Equipment Maintenance Log

Year: _____ Equipment Serial Number: _____ Location: _____

Procedure	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Daily Maintenance																															
1. **Check printer paper**																															
2. **Lysing Heater Temperature**																															
2.1 Digital display (114.0°C)																															
2.2 Manual Thermometer (112 - 116°C)																															
3. **Priming Heater**:**Temperature**																															
3.1 Digital display (72.5°C)																															
3.2 Manual Thermometer (72 - 73°C)																															
4. **Warming Heater Temperature**																															
4.1 Digital display (54°C)																															
4.2 Manual Thermometer (53.5 – 54.5°C)																															
5. **BD ProbeTec Temperature**																															
5.1 Instrument display (47.5 – 55.0°C)																															
6. Recharge pipettor (at end of day)																															
End of Day Clean-up																															
1. Dispose of Priming and Amplification microwells																															
2. Clean All countertops 10% JIK																															
3. Clean Metal Plates per SOP																															
4. Clean Lysing Rack and Lysing Heater Cover																															
5. Clean Lysing Heater, Priming and Warming Heater and BD ProbeTec ET Instrument																															
6. Clean Pipettor																															
INITIALS																															

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1.BECKMAN-COULTER AcT DIFF 2, SN:AE20804 Maintenance & Corrective Action Log

Date	Maintenance or Incident	Initials	Action & Comments	Initials	Date

VITAL LAB SELECTRA-E REAGENT TRACKING SHEET

Initials and Date	Reagent	Date and Time Prepared	Storage Condition	Expiration date and time	Date and Time Discarded	Initials and date

YRG CARE Infectious Diseases Laboratory

Purpose

Use of Expired Reagents		
SOP Number:	Total Number of Pages:	
Primary Author: E. Livant	Signature & Date:	
Reviewed by:	Signature & Date:	
Revision History:		
Distributed to:		
Approved by: Dr. K.G. Murugavel	Signature & Date:	
Distribution Date:	Effective Date:	Revision Due Date:

Purpose:
To establish a controlled method of using expired reagents in rare emergency situations until replacement reagents/products are procured, insuring prompt and accurate laboratory results.

Scope:
This procedure will apply any reagents or products with expiration dates used in any phase of any YRG CARE Infectious Disease Laboratory testing.

Rationale:

The use of expired reagents is not encouraged. Due to problems relating to shipping and customs, unexpired reagents may not always be available. Every effort should be made to avoid this situation through proper inventory control. It is although, sometimes in the best interests of the patient or study participant to use an expired reagent when the alternative is not generating a result. In order to ensure that expired reagents do not pose the risk of inaccurate results, extra attention must be given to Quality Control.

Comments:

1. Expired reagents can be used for training purposes as long as results are not reported.

2. Any expired reagent kept in the lab must be plainly labeled as “Expired” in large print.

3. Expired reagents may never be use for patient results if there are still unexpired reagents of the same type available.

Procedure:

1. Complete the certification form for use of Expired reagents/products (See Appendix A).

2. Perform necessary quality control as described in each test specific SOP. Omit this if it is a quality control product that is expired.

3. The form must be signed by a lab manager.

4. Indicate a new expiration date on the product which is two months beyond the original. The product must be reassessed each two months. This may be extended a maximum of 6 months after the original expiration date.

5. Discontinue the expired reagent as soon an unexpired reagents are acquired.

Appendix A

YRG CARE Infectious Diseases Laboratory Chennai, India

Certification for Use of Expired Reagents or Laboratory Products

On occasions, test systems and reagents that have been outdated must continue to be used. However, prior to continuing their use past the expiration date, the system must be tested to ascertain that they are functioning adequately. Proper quality control must be performed and documented. This is not to imply that use of expired reagents is in any way encouraged, but rather, to establish a controlled method of using correctly until replacement systems and reagents are procured.

Current Date:
Reason expired system/reagent needs be used:
Test System/Reagent:
Lot number:
Manufacturer Expiration Date:
Laboratory Extended Expiration Date: **(2 months after expiration)**

The attached documentation shows reasonable proof that the reagent is performing adequately, provided routine quality control limits are met.

APPROVAL

Printed Name_____

Signature_____

Date_____

Section 8: Monitoring Visits

In addition to visits from network staff, all laboratories will have periodic visits from external monitors. HPTN and MTN will be monitored by an NIH sponsored Clinical Site Monitoring Group (CSMG). These monitoring visits are essential to ensure that laboratories are meeting minimum standards of GCLP and provide useful information for laboratory improvement.

For most studies, there will be clinic monitors who visit the laboratory 4 times a year; these monitors may not have a laboratory background. These monitors will generally review specimen storage using a short list of random samples. They will make sure that these specimens are correctly stored in LDMS and then view that the specimens are physically present in the correct location. They may also review other aspects such as appropriate SSP or SOP for protocol testing in the lab, participation/accreditation certificates, of the laboratory as requested as indicated.

On a less frequent basis, potentially annually, the laboratory will receive a more comprehensive visit from a monitor who has laboratory experience. These visits will be conducted by PPD or appropriate DAIDS representatives. These visits will follow a general template and look at all aspects of GCLP. It is advisable to have one or more staff members who will guide the monitor around the laboratory. The monitor will want to speak with many of the laboratory staff and ask them questions.

Especially at international sites where there may be several languages used, it is important to make sure that you understand the requests of the monitor. You generally are given 24 hours to produce any document requested by the monitor but it is better to be able to produce them immediately. If the monitor has moved to another task when you locate any documentation, make every effort to give the document to the monitor.

At the end of the visit there will be a debriefing. At this meeting, the monitor will meet with the laboratory staff and discuss his/her findings. This is the time to produce any other documentation or discuss/clarify an items addressed. The monitor will then file a report, generally within 20 days, which will go to the site, appropriate network, and regulatory agencies (frequently DAIDS). The site will then need to respond to the report with corrective action if necessary and follow up as indicated, generally within 10 working days. It is important to note that the site may disagree with the findings of the monitor and that the regulatory agency will make the final decision on the issue.

The key to preparedness for monitoring visits is a constant and ongoing adherence to GCLP and good record keeping and filing. Sites are encouraged to conduct periodic internal audits to prepare themselves for external monitoring visits. More information can also be found in the HPTN and MTN MOP's.

HIV PREVENTION TRIALS NETWORK LABORATORY ASSESSMENT CHECKLIST

Date(s) of Assessment_____

1. GENERAL INFORMATION

Laboratory Name

Physical Address

Mailing Address

Telephone#_____
_____Fax# _____
_____
_____

Email: _____

Laboratory Owner

Laboratory Director

1.1. Is there an organizational chart for the lab? _____

1.2. Is there a list of personnel employed by this lab?_____

1.3. Have there been any recent changes in personnel? _____

1.4. What is the average length of time of employment for the technical staff? _____

1.5. Is there a list of tests performed by the lab? _____

1.6. Is all testing performed on site? _____

1.7. How long has the laboratory been at this location? _____

1.8. Are there multiply locations/testing sites for the lab? _____

1.9. Have there been any recent changes in the facilities or the equipment? (major repairs, new equipment, etc)? _____

_____

1.10. When was the lab lasted audited? By what group/agency? _____

2. GENERAL WALK THROUGH

Walk through the facility following the normal workflow as much as possible. Observe use of space, condition of the facility, personnel, equipment, safety precautions and any work currently in process. Make note of any areas that stand out for specific attention later. Identify who you will be working with the various areas of the laboratory assessment. _____

_____

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_____

3. GENERAL POLICIES

3.1. Written procedures

3.1.1. Is there a standard format for written procedures? _____

3.1.2. Is there an approval system for written procedures?_____

3.1.3. How are revised procedures handled?_____

3.2. Is there a written general quality control plan for the laboratory? _____

_____

3.2.1. Does the quality control plan include control material to be used and frequency of use?___

_____

3.3. Is there a written general quality assurance plan for the laboratory? _____

_____

3.3.1. Does the quality assurance plan include documentation of problems and corrective action taken?_____

_____

3.4. Does the laboratory participate in proficiency programs for each of the analytes tested?_____

3.4.1. Which programs? _____

3.4.2. Frequency?_____

3.4.3. How are the proficiency samples handled? _____

3.4.4. Who tests the proficiency samples? _____

3.4.5. Who reviews the proficiency results? _____

3.4.6. What action is taken if results are not within guidelines given? _____

3.5. Is there a general preventative maintenance policy for the laboratory?_____

_____

3.6. How is the preventative maintenance, monitoring or calibration handled for the following?

3.6.1. Pipettes_____

3.6.2. Thermometers _____

3.6.3. Centrifuges _____

3.6.4. Water _____

3.6.5. Clean/Dirty Glassware _____

3.6.6. Plate Washer _____

3.6.7. Plate Reader _____

3.6.8. Thermocycler _____

3.6.9. Incubators _____

3.6.10. Hoods _____

3.6.11. Bench Tops/Counters _____

3.7. For temperature sensitive equipment such as incubators, refrigerators and freezers:

3.7.1. Are the temperatures monitored and recorded daily? _____

3.7.2. Are the acceptable temperature ranges listed on the monitoring records? _____

3.7.3. What action is taken if the temperatures are out of range? _____

3.7.4. Is the room temperature monitored and recorded? _____

3.7.5. How are temperatures monitored when staff is not present? _____

3.7.6. Are liquid nitrogen freezers in use? Is the liquid nitrogen level monitored? _____

4. PERSONNEL ASSESSMENT

4.1. How are new personnel trained? Records? _____

_____

4.2. How are existing personnel trained on new equipment or procedures? _____

_____

4.3. How is the technical competency of the laboratory staff monitored? _____

_____

4.4. Are Department meetings held? Frequency? Who chairs? Records? _____

_____

5. SAMPLE MANAGEMENT

5.1. Collection of laboratory specimens:

5.1.1. Where are the specimens collected? _____

5.1.2. Who collects the specimens? _____

5.1.3. How are the specimens transported to the lab? _____

5.1.4. Are there guidelines for collection, labeling, preservation and handling? _____

5.1.5. Is there a unique identification for each patient specimen?_____

5.1.6. What procedures are in place to prevent mislabeling or mishandling? _____

5.2. Receipt of laboratory specimens:

5.2.1. How are the laboratory specimens received? _____

5.2.2. Are there guidelines for acceptation and rejection of specimens? _____

5.2.3. How is a rejected specimen handled? _____

5.2.4. What are the acceptance criteria for specimens? _____

5.3. Processing of laboratory specimens:

5.3.1. How are the specimens processed?_____

5.3.2. How are the specimens checked for correct labeling ? _____

5.3.3. How are the specimens stored pending testing?_____

5.3.4. Are specimens stored in a manner that assures specimen integrity prior to testing?_____

5.4. Testing of specimens:

5.4.1. How is the testing ordered? _____

5.4.2. Is there a pending worklist for the technologist to follow? _____

5.4.3. What checks are used to make sure a test request is not missed? _____

5.4.4. What checks are used to assure an ordered test was performed? _____

5.5. Is there a policy for retaining tested specimens?_____

5.6. Freezer Storage

5.6.1. How are specimens handled for long term storage?_____

5.6.2. What system is used to store and retrieve frozen specimens? _____

5.6.3. Are repeated freeze/thaws recorded by sample? _____

5.6.4. What is the acceptable temperature range for long term freezer storage of samples? _____

6. **TEST SYSTEMS**

6.1-6.10 The following questions apply in general to all tests.

6.1. Are there written procedures for each test performed? _____

6.2. Are all test modifications or changes approved by the laboratory director and included in the written procedure? _____

6.3. Are procedures current and reviewed by the laboratory director? Date of last review? _____

6.4. If package inserts are used for procedures, are they supplemented with specific instructions where needed? _____

6.5. What mechanism is used to determine if the procedures are being followed? _____

6.6. What action is taken if patient test values are outside the test systems linear/reportable range? _____

6.7. Critical Values:

6.7.1. Is there a critical value policy? _____

6.7.2. Are critical values posted? _____

6.7.3. What action is taken for critical values? _____

6.8. If dilutions are made on a sample, are the dilutions and calculations recorded on the worksheets? _____

6.9. How are exceptions to the written procedures handled? _____

6.10. What steps are taken to add a new test method or instrument to the lab? _____

6.11-6.21 The following questions are test specific

6.11. Do the test records identify who performed the test? _____

6.12. Are the original test records stored? For how long?_____

6.13. Are the QC requirements listed in the procedures? _____

6.14. Are a minimum of two levels of controls run each day of testing? _____

6.15. How are QC values determined? _____

6.16. Are QC results documented and reviewed?_____

6.17. Are calibration requirements documented and followed? _____

6.18. What is the frequency of calibration? _____

6.19. Are maintenance requirements documented and followed? _____

6.20. Are problems and corrective action documented for each test or instrument? _____

6.21. Are the calibration, maintenance and corrective action records reviewed periodically and signed?_____

7. **RESULTS**

7.1. Who reviews the test results? _____

7.2. How are test results reviewed? Does the reviewer sign/initial the final results? _____

7.3. Are individual results reviewed for correlation with each other (ie hgb/hct)? _____

7.4. What parameters are reviewed for correlation of patient results? _____

7.5. What action is taken if individual results do not correlate with each other? _____

7.6. In what form(s) are results released from the laboratory?_____

7.7. Does the laboratory result report include reference ranges? age, gender specific? _____

7.8. How are critical values handled? _____

7.9. Does the report include a comments section? _____

7.10. What mechanism is used to detect reporting errors? _____

7.11. How are changes to reported results handled? _____

7.12. Is an exact copy of the original report kept by the lab? What form (hardcopy,

computerized)? For how long? _____

8. **RECORDS MANAGEMENT**

8.1. Can a specimen be tracked from the test requisition through the test reporting? _____

8.2. How is the records storage system set up? _____

8.3. Are the following records maintained and readily retrievable?

8.3.1. Requisitions/Test Orders_____

8.3.2. Worksheets, Instrument printouts _____

8.3.3. Test results or reports_____

8.3.4. QC records _____

8.4. How long are the above records maintained?_____

9. **REAGENTS/SUPPLIES**

9.1. How are reagents and supplies ordered? _____

9.2. Are there problems with acquiring supplies?_____

9.3. How are reagents/supplies received/stored?_____

9.4. How are expired reagents handled? _____

9.5. Are reagents properly labeled? _____
(name, concentration, storage requirements, date received/prepared, date in use, expiration date)

10. **LABORATORY INFORMATION SYSTEMS (LIS)**

10.1. Is there a mechanism to prevent alteration or destruction to major computer programs? (anti-virus programs) _____

10.2. Are there specific user levels within the LIS? (security levels) _____

10.3. Is there a policy on who is authorized to change information within the LIS? _____

10.4. Is there a system in place to prevent loss of results in case of hardware or software failure (backup/recovery)?_____

10.5.

10.6. How often is backup performed? _____

10.7. What type of media is used for backup and where are the backup copies kept? _____

10.8. Is there scheduled maintenance on the hardware and software?_____

10.9. Is there a contingency plan for reporting results if the LIS fails? _____

10.10. Archiving:

10.10.1. Are results periodically archived? _____

10.10.2. Can the archived results be retrieved? _____

10.10.3. When are results archived? _____

10.10.4. How long are the archived results maintained?_____

11. **FACILITY**

11.1. Are the environmental conditions (lighting, electricity, temperature, ventilation, space etc) appropriate for the work performed?_____

11.2. In case of an electrical outage what steps are taken?_____

11.3. What type of back-up power supply is available?_____

11.4. How would samples be protected during an outage?_____

11.5. Is the electrical system adequately protected against electrical power interruptions and surges?_____

12. **SHIPPING/TRANSPORTING**

12.1. Is the laboratory involved in shipping of dangerous goods? _____

12.2. Is there a written policy for shipping dangerous goods?_____

12.3. Who handles the shipping of dangerous goods? _____

12.4. What training is required for employees involved in the shipping of dangerous goods? ___

12.5. Are certification records available?

13. **SAFETY**

13.1. Is there a biohazard safety policy?

13.2. Is personal protective gear available and used?

13.3. Is there a chemical hygiene plan?

13.4. Is there a hazardous chemical policy? MSDS available?

13.5. How is biohazard waste handled?

13.6. Are there periodic inspections of the electrical system?

13.7. Is the laboratory facility and workflow designed to enhance employee safety?

13.8. Is fire-fighting equipment (extinguishers) available? In-date?

14. **COMMENTS**

14.1. What are your main concerns for the laboratory at this time?

14.2. What would be of the most benefit for the lab at this time?

Monitoring of Stored Specimens in the Laboratory

The monitoring of stored laboratory specimens by PPD for the HPTN protocol trials will provide the CL and DAIDS with useful information about the specimen management by sites. For all the international and domestic sites involved in on-going HPTN protocols, the clinical monitoring visits by PPD should include a review of specimen management procedures. For this purpose, prior to a monitoring visit PPD should contact the protocol specialist at SCHARP and obtain a list of random patient ID# (PTID) of which the monitor would choose of 5 PTID's for each protocol (10 PTID's for HPTN 035- see below for details) for audit. PTIDs to be reviewed at each visit will not be pre-announced to the site.

During the monitoring visit, the monitor would look for the following information:

1. Documentation of primary specimens received for each of these PTID, including the tracking forms and/or local lab requisition slips.
2. All document(s) indicating the laboratory test procedures conducted for that particular PTID. This should include copies of the Laboratory section of the SSP.
3. Demonstration in LDMS:
   a. receipt of primary specimen(s)
   b. existence of required aliquot(s)
   c. storage of required aliquot(s)
   d. shipment of required aliquot(s)
   e. documentation of reason(s) for deviation from protocol requirements
4. Aliquots from one of the randomly chosen PTIDs to show that the aliquots are properly labeled.

Special instructions for HPTN 035:

For the 035 study, the monitor is required to check 5 specimens from each category of stored specimens including:

- Follow-up Plasma/Enrollment Plasma
- Gram Stain slides/GUD Swabs

It is critical that the monitor prioritize the checking of 035 Follow-up plasma sample (s) included on the list from SCHARP. For security purposes, all seroconversion samples will appear in the SCHARP list as "Follow-up plasma samples". Next in priority is to check for the "Enrollment Plasma Samples", then "Gram Stain" specimens, and then, check for any "GUD Swab" specimens listed. Since stored plasma specimens are required to confirm 035 primary study endpoint, PPD should prioritize the checking of 'Follow-up' and 'Enrollment' plasma samples during their monitoring visits.

Also, it will be important to check the number of aliquots stored for each of the specimen types (as per the SSP). For example, for each 'Follow-up plasma sample" the monitor should make sure that the site has at-least four 0.5ml aliquots of the plasma specimen.

Reporting of findings from the audit:

For the specimens verified at the site, the monitoring report should only show information on Specimen ID, Specimen collection Date, specimen type and storage location. Information on visit type (enrollment or follow-up) or visit number should **not** be included. in the monitoring report.

The HPTN CL would review the monitoring report for any problems noted during this review. This will help the CL determine if sites need extra training in the use of the LDMS as well as ensure that the appropriate samples are being maintained for the protocols.

Division of AIDS
Clinical Research Operations and Monitoring Center

LABORATORY ASSESSMENT VISIT REPORT

Protocol Title:

Study Number:

Name & Location of Laboratory:

Laboratory Director:

Biosafety Level of Laboratory:
(as applicable)

Date Last Monitored:

Grant Principal Investigator:

Grant Number:

DAIDS Program Officer:

DAIDS Medical Officer:

Date(s) of Visit:

Conducted by:

TABLE OF CONTENTS

SUMMARY OF LABORATORY DEBRIEFING, SIGNIFICANT FINDINGS AND RECOMMENDATIONS .....

STATUS OF PAST FINDINGS .....
Delete if this is first pre-trial site visit

LABORATORY ASSESSMENT .....

SUMMARY OF LABORATORY DEBRIEFING, SIGNIFICANT FINDINGS, AND RECOMMENDATIONS

Suggested format for standardizing style - document summary of findings with headings that are ordered and correspond to Table of Contents and sections of the report.

For Pre-trial assessments, use the following format:

I. LABORATORY ASSESSMENT

A. **Requirements or Major Deficiencies** that must be corrected prior to trial initiation.

1.
2.
3.

B. **Recommendations or Minor Deficiencies** that can be addressed on an ongoing basis.

1.
2.
3.

For all other assessments, use this format:

I. LABORATORY ASSESSMENT

Summarize findings

Recommendations or Corrective Actions

Note these individually

STATUS OF PAST FINDINGS

Delete this section if this is the first laboratory assessment

DIVISION OF AIDS
CLINICAL RESEARCH OPERATIONS AND MONITORING CENTER

LABORATORY ASSESSMENT

Instructions: Indicate if the tasks and topics listed were reviewed and assessed and are adequate. Provide detailed comments as applicable. Indicate items that were not reviewed or assessed by checking 'No' or by leaving the fields blank; describe in the Comments sections. Indicate items that are not applicable in the Comments sections.

Laboratory Activities

Hematology	Yes ☐	No ☐	Comments ☐
Flow Cytometry	Yes ☐	No ☐	Comments ☐
Chemistry	Yes ☐	No ☐	Comments ☐
Urine Analysis	Yes ☐	No ☐	Comments ☐
Protocol-Specific Assays	Yes ☐	No ☐	Comments ☐
Microbiology	Yes ☐	No ☐	Comments ☐
Virology	Yes ☐	No ☐	Comments ☐
PBMC Processing	Yes ☐	No ☐	Comments ☐
Immunology/Serology	Yes ☐	No ☐	Comments ☐
Specimen Storage	Yes ☐	No ☐	Comments ☐
Specimen Shipping	Yes ☐	No ☐	Comments ☐
Other	Yes ☐	No ☐	Comments ☐

Comments:

External Quality Assurance (EQA)

A.	**Does the laboratory participate in any external proficiency testing? (If "Yes", list the tests involved, frequency, proficiency program provider, and a summary of performance.)**	Yes ☐ No ☐ Comments ☐

Comments:

I. Organization and Personnel

A.	**Is an organizational chart for laboratory personnel present?**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Is job description/delegation of duties documentation present?**	Yes ☐ No ☐ Comments ☐

Comments:

C.	**For each laboratory position, is there a documented profile that list requirements such as education, experience, and certification/license requirements? If "Yes", please list all position requirements in the "Comments" section.**	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Are education records maintained for all laboratory employees?**	Yes ☐ No ☐ Comments ☐

Comments:

E.	**Are training records for personnel kept on file?**	Yes ☐ No ☐ Comments ☐

Comments:

F.	**Has the laboratory staff undergone Good Laboratory Practice (GLP) training?**	Yes ☐ No ☐ Comments ☐

Comments:

G.	**Personnel Competency**	
1.	Is documentation maintained, indicating the laboratory has assessed the competency of each employee to perform his or her assigned duties? If "Yes", please report the methods utilized to assess competency in the "Comments" section.	Yes ☐ No ☐ Comments ☐

Comments:

G.	**Has the laboratory been certified by any regulatory agency? If "Yes", please list the agency and date of certification.**	Yes ☐ No ☐ Comments ☐

Regulatory Agency	Dates of Certification

II. Equipment

A.	**Laboratory Equipment**	
Verify following as it applies to the laboratory equipment:		
1.	Are freezers present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are temperature readings taken/documented? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	c. Have tolerance limits been established/documented for temperature readings?	Yes ☐ No ☐ Comments ☐
	d. Is there documentation of corrective actions taken, in response to out-of-range values?	Yes ☐ No ☐ Comments ☐
2.	Are refrigerators present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are temperature readings taken/documented? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	c. Have tolerance limits been established/documented for temperature readings?	Yes ☐ No ☐ Comments ☐
	d. Is there documentation of corrective actions taken, in response to out-of-range values?	Yes ☐ No ☐ Comments ☐
3.	Are liquid nitrogen freezers present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are temperature readings or nitrogen levels taken/documented? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐

	c. Have tolerance limits been established/documented for temperature readings?	Yes ☐ No ☐ Comments ☐
	d. Is there documentation of corrective actions taken, in response to out-of-range values?	Yes ☐ No ☐ Comments ☐
4.	Are incubators present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are temperature readings taken/documented? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	c. Have tolerance limits been established/documented for temperature readings?	Yes ☐ No ☐ Comments ☐
	d. Is there documentation of corrective actions taken, in response to out-of-range values?	Yes ☐ No ☐ Comments ☐
5.	Are water baths present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are temperature readings taken/documented? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	c. Have tolerance limits been established/documented for temperature readings?	Yes ☐ No ☐ Comments ☐
	d. Is there documentation of corrective actions taken, in response to out-of-range values?	Yes ☐ No ☐ Comments ☐
6.	Are centrifuges present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Is calibration performed/documented for each centrifuge? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
7.	Are biosafety cabinets/laminar air flow hoods present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Has each cabinet/hood been certified? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
8.	Is chemistry instrumentation present? If "Yes", please report the manufacturer/model in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are calibration procedures performed, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐
9.	Is hematology instrumentation present? If "Yes", please report the manufacturer/model in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are calibration procedures performed, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐

10.	Is flow cytometry instrumentation present? If "Yes", please report the manufacturer/model in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are calibration procedures performed, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐
11.	Is PCR/molecular testing equipment present? If "Yes", please report the manufacturer/model in the "Comments" section.	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
12.	Are pipettors present?	Yes ☐ No ☐ Comments ☐
	a. Are calibration procedures performed for all pipettors? If "Yes", please report the frequency in the "Comments" section.	Yes ☐ No ☐ Comments ☐
13.	Are thermometers present?	Yes ☐ No ☐ Comments ☐
	a. Is a known standard thermometric device available (NIST certified)?	Yes ☐ No ☐ Comments ☐
	b. Have all non-certified thermometers been tested against a standard device?	Yes ☐ No ☐ Comments ☐
14.	Are balances present?	Yes ☐ No ☐ Comments ☐
	a. Are calibration procedures performed, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐
15.	Is immunology testing equipment present?	Yes ☐ No ☐ Comments ☐
	a. Are Preventative Maintenance activities performed/documented?	Yes ☐ No ☐ Comments ☐
	b. Are calibration procedures performed, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐
16.	Are additional equipment used for protocol-related assays present? If "Yes", please describe in the "Comments" section.	Yes ☐ No ☐ Comments ☐
17.	Is all laboratory equipment listed on an inventory document?	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Is there a written policy/procedure in place, explaining how temperatures are monitored during the absence of laboratory staff?**	Yes ☐ No ☐ Comments ☐

Comments:

C.	**Supervisor or designee has reviewed and signed maintenance, repair, and calibration records monthly?**	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Are there documented PM and calibration plans for the laboratory's equipment?**	Yes ☐ No ☐ Comments ☐

Comments:

E. Are calibration materials stored, as required by the manufacturer?	Yes ☐ No ☐ Comments ☐

Comments:

F. Are calibration materials properly labeled, indicating content and calibration value?	Yes ☐ No ☐ Comments ☐

Comments:

G. Are there records to verify that a back-up generator system is in place and operational?	Yes ☐ No ☐ Comments ☐

Comments:

III. Testing Facilities Operation

A. Is there a list of all testing activities performed in the laboratory?	Yes ☐ No ☐ Comments ☐

Comments:

B. Standard Operating Procedures

Written Procedure Name	Present	Annual Review Completed by Lab Director/Designee?	Lab Director/Designee Signature Present?
1.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
2.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
3.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
4.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
5.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
6.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
7.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐

8.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
9.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐
10.	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐	Yes ☐ No ☐ Comments ☐

Comments:

C. Is there a written document control plan that addresses topics such as procedural relevance, authorization process, annual reviews, and discontinuation of procedures?	Yes ☐ No ☐ Comments ☐

Comments:

D. Are the laboratory's SOPs reviewed for accuracy and relevance on an annual basis?	Yes ☐ No ☐ Comments ☐

Comments:

E. Does the laboratory have a system of documenting that all personnel are knowledgeable of the contents of the laboratory's SOPs?	Yes ☐ No ☐ Comments ☐

Comments:

F. Are the laboratory's SOPs available in the work area?	Yes ☐ No ☐ Comments ☐

Comments:

G. Are old versions (retired) of SOPs identified as retired and archived in the laboratory?	Yes ☐ No ☐ Comments ☐

Comments:

H. Is there a list of assay turnaround times present?	Yes ☐ No ☐ Comments ☐

Comments:

I. Does the laboratory have a policy for employees to communicate concerns, regarding testing quality or laboratory safety to management?	Yes ☐ No ☐ Comments ☐

Comments:

IV. Test and Control Articles

A. Qualitative/Quantitative Tests

Name of Assay	QC Frequency
1. Is there a written Quality Control program that clearly defines procedures for monitoring analytic performance, including establishment of tolerance limits, number and frequency of control tests, corrective action based on Quality Control data, and related information?	Yes ☐ No ☐ Comments ☐
2. Are logs present, documenting control results assayed with each test, as described in the specific assay procedure?	Yes ☐ No ☐ Comments ☐
3. Does the technologist performing the QC initial the logs?	Yes ☐ No ☐ Comments ☐
4. Are appropriate charts utilized to document QC data (i.e. Levy Jennings charts)?	Yes ☐ No ☐ Comments ☐
5. Has a supervisor/designee reviewed and signed Levy Jennings charts? (If "Yes", note the frequency in the "Comments" section.)	Yes ☐ No ☐ Comments ☐
6. Has a supervisor/designee reviewed and signed all QC logs? (If "Yes", note the frequency in the "Comments" section.)	Yes ☐ No ☐ Comments ☐
7. Are all QC documents available for the past two years?	Yes ☐ No ☐ Comments ☐
8. For quantitative tests, are control materials at more than one level used?	Yes ☐ No ☐ Comments ☐
9. For qualitative tests, is a positive and negative control included with each run?	Yes ☐ No ☐ Comments ☐

Comments:

B. Quality Control Failure/Corrective Action

1. Is there a Corrective Action Log present, documenting resolution of QC failures?	Yes ☐ No ☐ Comments ☐
2. Has a supervisor/designee reviewed and signed the Corrective Action Log? (If "Yes", note the frequency in the "Comments" section.)	Yes ☐ No ☐ Comments ☐

Comments:

C.	QC Materials	
1.	Are all Quality Control materials dated within the manufacturer's assigned expiration dates?	Yes ☐ No ☐ Comments ☐
2.	Are all Quality Control materials properly stored, as required by the manufacturer?	Yes ☐ No ☐ Comments ☐
3.	If calibrators are used as controls, are they from a different lot number than those used to calibrate the method?	Yes ☐ No ☐ Comments ☐

Comments:

D.	Reagent/Testing Kits	
1.	Are all reagents/testing kits within the manufacturer's assigned expiration dates?	Yes ☐ No ☐ Comments ☐
2.	Are all reagents/testing kits properly stored, as described by the manufacturer?	Yes ☐ No ☐ Comments ☐
3.	Are all reagents/solutions properly labeled, to indicate identity, lot number, storage requirement, date prepared/reconstituted, and expiration date?	Yes ☐ No ☐ Comments ☐
4.	Is there an inventory control system in operation?	Yes ☐ No ☐ Comments ☐

Comments:

E.	Water Quality	
1.	Does the laboratory testing require specific water types, for certain testing procedures? If "Yes", please describe in the "Comments" section.	Yes ☐ No ☐ Comments ☐
2.	If specific water types are utilized, is there a documented policy that defines standards and frequency of water testing?	Yes ☐ No ☐ Comments ☐

Comments:

F.	Parallel Testing	
1.	HIV EIA Assay – Is there documentation that three patient samples are tested with each new lot of reagents with reproducible results?	Yes ☐ No ☐ Comments ☐
2.	HIV RNA PCR – Is there documentation that three patient samples (non-detected, mid-range, and high range) are tested with each new lot? Are results within a prescribed range of the results, obtained with the previous lot number?	Yes ☐ No ☐ Comments ☐
3.	Qualitative PCR Assays – Is there documentation that three samples were tested with each new lot of reagents with reproducible results?	Yes ☐ No ☐ Comments ☐

4.	CBCFBC – Are new lot controls run in parallel with the previous lot, before being put into use?	Yes ☐ No ☐ Comments ☐
5.	Chemistry Controls – Are new lots of controls run in parallel with the previous lot of controls, to establish a mean and acceptable standard deviation?	Yes ☐ No ☐ Comments ☐
6.	CD4/CD8 – Are at least two patient specimens (CD4:CD8 ratio <1.0 and >1.0) run in parallel with reproducible results?	Yes ☐ No ☐ Comments ☐
7.	Does the supervisor or director sign validity checks for each parallel test?	Yes ☐ No ☐ Comments ☐

Comments:

G.	**Culture Media**	
1	Is a media QC log present?	Yes ☐ No ☐ Comments ☐
2.	Does a technologist initial the media QC log?	Yes ☐ No ☐ Comments ☐
3.	Is a QC Failure/Corrective Action Log present for media?	Yes ☐ No ☐ Comments ☐
4.	Are both logs (QC and QC Failure/Corrective Action) reviewed and signed by supervisor/designee monthly?	Yes ☐ No ☐ Comments ☐
5.	Is the media in use expired?	Yes ☐ No ☐ Comments ☐

Comments:

H.	**Staining Procedures**	
1.	Is a control slide stained weekly with documented observations initialed by a technologist?	Yes ☐ No ☐ Comments ☐
2.	Is a Corrective Action Log present for staining QC?	Yes ☐ No ☐ Comments ☐
3.	Are both logs reviewed and signed by supervisor/designee monthly?	Yes ☐ No ☐ Comments ☐

Comments:

V. Verification of Performance Specifications

A.	**Has the laboratory verified or established and documented analytic accuracy and precision for each test?**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Has the laboratory verified or established and documented the analytic sensitivity (lower detection limit) of each assay, as applicable?**	Yes ☐ No ☐ Comments ☐

Comments:

C.	**Has the laboratory verified or established and documented analytic interferences for each test?**	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Has the laboratory verified or established and documented reference intervals for each test?**	Yes ☐ No ☐ Comments ☐

Comments:

VI. Records and Reports

A.	**Are copies of network lab-specific manuals, protocols, and appendices available?**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Specimen Tracking Forms/Requisitions**	
1.	Are forms readily available for at least two years?	Yes ☐ No ☐ Comments ☐
2.	Are the forms retrievable for the entire protocol?	Yes ☐ No ☐ Comments ☐

Comments:

C.	**Is specimen chain of custody adequately documented?**	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Is there a list of places laboratory results are reported?**	Yes ☐ No ☐ Comments ☐

Comments:

E.	**Where appropriate, are analyte results reported with accompanying reference intervals?**	Yes ☐ No ☐ Comments ☐

Comments:

F.	**Do the laboratory reports identify the laboratory performing the testing?**	Yes ☐ No ☐ Comments ☐

Comments:

G.	**Does the laboratory archive result data (result printouts, etc.)? If "Yes", please explain in the "Comments" section how archiving is accomplished, and how long data is archived.**	Yes ☐ No ☐ Comments ☐

Comments:

H.	**Are the archived records accessible to only authorized personnel?**	Yes ☐ No ☐ Comments ☐

Comments:

VII. Physical Facilities

1.	Is there adequate, conveniently located space so the quality of work and safety of personnel are not compromised?	Yes ☐ No ☐ Comments ☐
2.	Are the ventilation and humidity adequately controlled in all areas?	Yes ☐ No ☐ Comments ☐
3.	Are ambient room temperature readings taken/documented?	Yes ☐ No ☐ Comments ☐
	a. Have tolerance limits been established/documented for ambient room temperature?	Yes ☐ No ☐ Comments ☐

Comments:

VIII. Specimen Transport and Management

A.	**Is a manual containing instructions for specimen collection in the laboratory and areas dedicated for specimen collection?**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Is there a documented policy/procedure for identifying and assessing the quality of specimens received in the laboratory?**	Yes ☐ No ☐ Comments ☐

Comments:

C.	**Specimen Transport**	
1.	Is there a policy/procedure in place for transporting samples (transported in a sturdy, non-breakable, closable container labeled "biohazard")?	Yes ☐ No ☐ Comments ☐

2.	Does the document address transport within the facility?	Yes ☐ No ☐ Comments ☐
3.	Does the document address transportation between off-site clinics and the laboratory?	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Are wet specimens (blood, urine, body fluids, etc.) retained for potential re-evaluation? If "Yes", explain in the "Comments" section.**	Yes ☐ No ☐ Comments ☐

Comments:

E.	**Shipping Certification/Training**	
1.	Is there a training plan in place for shipping certification?	Yes ☐ No ☐ Comments ☐
2.	Is there documentation of persons trained for shipping?	Yes ☐ No ☐ Comments ☐
3.	Are shipping certifications renewed every 2 years?	Yes ☐ No ☐ Comments ☐
4.	Is there a policy in place for shipping samples internationally?	Yes ☐ No ☐ Comments ☐

Comments:

IX. Personnel Safety

A.	**Safety-Related Incidents**	
1.	Is there documentation of all safety-related incidents signed monthly by laboratory director or designee?	Yes ☐ No ☐ Comments ☐
2.	Has an evaluation of these incidents been incorporated into the laboratory's Quality Management program, to avoid recurrence?	Yes ☐ No ☐ Comments ☐

Comments:

B.	**Material Safety Data Sheets (MSDS)**	
1.	Are MSDS on file?	Yes ☐ No ☐ Comments ☐
2.	Are MSDS readily available to all laboratory personnel?	Yes ☐ No ☐ Comments ☐

Comments:

C. Is there documented participation of laboratory personnel in a safety training program? If "Yes", list who provides the training, and what is the frequency of the training?	Yes ☐ No ☐ Comments ☐

Comments:

D. Safety Policies		
1.	Is a written Standard Precautions Policy available?	Yes ☐ No ☐ Comments ☐
2.	Is a written Chemical Hygiene/Hazardous Materials Plan available?	Yes ☐ No ☐ Comments ☐
3.	Is there a written policy for the handling and disposal of biohazardous materials and regulated medical waste?	Yes ☐ No ☐ Comments ☐
4.	Are safety policies and procedures readily available to all staff?	Yes ☐ No ☐ Comments ☐
5.	Is there evidence of at least annual review of safety policies and procedures by the laboratory director/designee?	Yes ☐ No ☐ Comments ☐
6.	Are policies, procedures, and practices in place for use of liquid nitrogen?	Yes ☐ No ☐ Comments ☐
7.	Are policies, procedures, and practices in place for use of dry ice (solid carbon dioxide)?	Yes ☐ No ☐ Comments ☐

Comments:

E. Is safety equipment such as eyewashes, safety showers, fire extinguishers, fire blankets, and sharps containers present in the lab?	Yes ☐ No ☐ Comments ☐

Comments:

F. Personal Protective Equipment (PPE)		
1.	Is Personal Protective Equipment (gloves, gowns, masks, eye protectors, etc.) available to and utilized by laboratory staff? (If "No", explain in "Comments" section.)	Yes ☐ No ☐ Comments ☐
2.	Is Personal Protective Equipment maintained in a sanitary and reliable condition in all technical work areas, in which blood and body substances are handled, and in circumstances during which exposure is likely to occur?	Yes ☐ No ☐ Comments ☐

Comments:

X. Laboratory Information Systems (LIS)

A.	**Is an LIS utilized in this laboratory? (If "No", the rest of Section XI is "N/A".)**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**LIS**	
1.	Is documented validation data present for the LIS?	Yes ☐ No ☐ Comments ☐
2.	Can accurate and complete copies be generated by the LIS?	Yes ☐ No ☐ Comments ☐
3.	Are computer time-stamped audit trails used by the LIS?	Yes ☐ No ☐ Comments ☐
4.	Is system access limited to authorized individuals?	Yes ☐ No ☐ Comments ☐
5.	Is there a written SOP for the operation of the LIS present?	Yes ☐ No ☐ Comments ☐
6.	Is there a back-up system for the LIS?	Yes ☐ No ☐ Comments ☐

Comments:

XI. Laboratory Data Management System (LDMS)

A.	**Does this laboratory facility contain a LDMS? If "No", disregard the rest of Section XII, and explain in the "Comments" section how specimen storage/shipping data is maintained.**	Yes ☐ No ☐ Comments ☐

Comments:

B.	**LDMS Reports Obtained By the Monitor:**	
1.	Primary Specimens Received Report	Yes ☐ No ☐ Comments ☐
2.	Storage Detail Report	Yes ☐ No ☐ Comments ☐
3.	Shipping Report	Yes ☐ No ☐ Comments ☐

Comments:

C. Specimen Verification

1	Can the PID, date, protocol, derivative, and additive for specimens be verified with the LDMS?	Yes ☐ No ☐ Comments ☐
2.	Is the laboratory staff able to demonstrate specimen storage locations in LDMS?	Yes ☐ No ☐ Comments ☐
3.	Does the LDMS accurately reflect the number, type, and volume of all specimen aliquots as well as their storage location and shipping record?	Yes ☐ No ☐ Comments ☐
4.	Can the physical presence of specimens be verified with the LDMS Storage Detail Report?	Yes ☐ No ☐ Comments ☐

Comments:

D.	**Is the LDMS manual available in laboratory?**	Yes ☐ No ☐ Comments ☐

Comments:

E.	**LDMS Back-Up**	
1.	Is the LDMS backed up weekly?	Yes ☐ No ☐ Comments ☐
2.	Is the LDMS back-up disk stored in different location than LDMS computer?	Yes ☐ No ☐ Comments ☐

Comments:

XII. Quality Management

1.	Does the laboratory have a Quality Assurance/Quality Management program?	Yes ☐ No ☐ Comments ☐
2.	Does the program follow a documented operational plan, designed to monitor, assess, and (when indicated) correct problems identified in pre-analytic, analytic, and post-analytic systems as well as general laboratory systems?	Yes ☐ No ☐ Comments ☐
3.	Are key indicators of quality monitored and evaluated, to detect problems and opportunities for improvement?	Yes ☐ No ☐ Comments ☐
4.	Are appropriate corrective action and/or preventive actions taken, when opportunities for improvement are identified?	Yes ☐ No ☐ Comments ☐

Comments:

XIII. Vertical Audit of SOP/Practice

A.	Pre-Test Specimen Handling	
1.	Are specimens submitted for testing, as required by the SOP?	Yes ☐ No ☐ Comments ☐
2.	Are specimens maintained at appropriate conditions (e.g. temperature), until testing can be performed?	Yes ☐ No ☐ Comments ☐
3.	Are specimens thawed and counted, as required by the SOP? (Note method of cell counting in the "Comments" section.)	Yes ☐ No ☐ Comments ☐
	a. For hemocytometer counted samples: Are specimens counted in duplicate?	Yes ☐ No ☐ Comments ☐
4.	Is cell viability and recovery documented for specimens?	Yes ☐ No ☐ Comments ☐

Comments:

B.	Reagent Preparation and Storage	
1.	Are reagents prepared, in accordance with the SOP?	Yes ☐ No ☐ Comments ☐
2.	Are reagents maintained at appropriate conditions, until testing can be performed?	Yes ☐ No ☐ Comments ☐

Comments:

C.	Test Set-Up	
1.	Are tubes/plates pre-labeled prior to testing? If so, how far in advance?	Yes ☐ No ☐ Comments ☐
2.	Are tubes/plates labeled appropriately with sufficient identification, to prevent mix-up?	Yes ☐ No ☐ Comments ☐
3.	Is appropriate equipment (e.g. pipettes, vortex mixer, etc.) available at the start of the procedure, to avoid delay?	Yes ☐ No ☐ Comments ☐

Comments:

D.	Stimulation/Incubation Phase	
1.	Are appropriate conditions maintained to perform the assay (e.g. sterile, biohazard containment)?	Yes ☐ No ☐ Comments ☐
2.	Are reagents and samples added in the appropriate order, and at appropriate times?	Yes ☐ No ☐ Comments ☐
3.	Are appropriate controls (positive and negative) available and tested?	Yes ☐ No ☐ Comments ☐
4.	Is an incubation time required?	Yes ☐ No ☐ Comments ☐

	a. Is it performed appropriately?	Yes ☐ No ☐ Comments ☐
5.	Are additional steps followed as defined in the SOP?	Yes ☐ No ☐ Comments ☐
6.	Are samples maintained under appropriate conditions until analysis?	Yes ☐ No ☐ Comments ☐

Comments:

E.	**Analysis Phase**	
1.	Is an analyzer required for this phase? (If "No", skip to Question 2.)	Yes ☐ No ☐ Comments ☐
	a. Is the analyzer set up, as required by the SOP?	Yes ☐ No ☐ Comments ☐
	b. Are appropriate controls and, when applicable, calibrators available and tested?	Yes ☐ No ☐ Comments ☐
	c. Are samples analyzed, as defined by the SOP?	Yes ☐ No ☐ Comments ☐
	d. If required by SOP, are appropriate "blanking" samples utilized?	Yes ☐ No ☐ Comments ☐
	e. If required by SOP, are analysis regions set on control samples?	Yes ☐ No ☐ Comments ☐
	f. Are specimens analyzed within the timeframe, as defined in the SOP?	Yes ☐ No ☐ Comments ☐
2.	Are samples analyzed by manual methods? (If "No", skip to Section F.)	Yes ☐ No ☐ Comments ☐
	a. Are specimens analyzed within the timeframe, as defined in the SOP?	Yes ☐ No ☐ Comments ☐

Comments:

F.	**Calculations and Result Reporting**	
1.	If manual calculations are performed, is the derivation of the final result available?	Yes ☐ No ☐ Comments ☐
2.	Are results transmitted from analyzer to a central LIS?	Yes ☐ No ☐ Comments ☐
3.	Do the results obtained by the analyzer, match those in the LIS?	Yes ☐ No ☐ Comments ☐
4.	Are results verified by alternate personnel?	Yes ☐ No ☐ Comments ☐
5.	Are results reported as defined in the SOP?	Yes ☐ No ☐ Comments ☐

Comments:

Section 9: Backup

Each laboratory should consider back-up arrangements for all tests that are performed and the staff that perform them. It is not advisable to have only one person who routinely performs a test or procedure. Back-up staff that does not perform a test or procedure regularly should rotate onto that test or procedure periodically so that they can maintain competency.

If possible, it is advisable to have secondary equipment within your laboratory that can serve as a backup in case problems arise with the primary equipment. Secondary equipment must have all necessary method validation, maintenance, and quality control (QC) as the primary instrument. Before equipment is classified as a back-up, comparison studies should be performed and periodic split samples should be run between each instrument to document ongoing correlation.

In cases where it is not possible to have internal secondary equipment, it may be necessary to designate an external laboratory to serve as a back-up. HPTN and MTN have requirements for use of external laboratories either for routine testing or in cases of back-up. These include testing blinded specimens, monitoring of QC, external proficiency testing, archiving of lab documents, and visits from the Central Laboratory representatives. Refer to either network MOP for more information.

For laboratories/sites that use non affiliated laboratories at any time during a HPTN protocol for safety determinations such as an instrument failure (ie CBC/chemistries) , the Central laboratory is recommending the implementation of the following on a monthly or as needed basis:

Two samples (a known normal and an abnormal) should be submitted in duplicate to the laboratory. The results should compare within Alternatively, a preserved sample with known values may be submitted. The results should fall within the ranges indicated by the company.

The site is responsible for documenting and maintaining these results. These results should be faxed monthly to the Central Laboratory for review.

Quality Control records and Levy Jennings Charts for laboratory safety determinations must be submitted by fax monthly to the Central laboratory.

Section 10: Hematology

1. Overview: Hematology is the study of the cellular and certain chemical elements of blood. The complete blood count (CBC) including white blood cells is the primary test performed in a hematology laboratory. Numerous HPTN/MTN studies require a CBC to screen for healthy study participants and monitor potential adverse events.

   Hematology testing requires personnel with an adequate level of training to be able to identify abnormal or erroneous results and take appropriate corrective action. It is advisable that a pathologist be available to review highly abnormal slides and to assist with difficult specimens. Areas with endemic blood parasites such as malaria will have additional considerations. Following is a very brief overview of hematology, followed by some examples of hematology SOP's and forms.

2. Specimen: most hematology testing will be done with fresh EDTA whole blood. Specimens should be checked manually with applicator sticks for clots unless the instrument utilized has clot detection; some clots cannot be detected by visually inspecting the specimens. Clotted samples can interfere with analyzer performance, may cause erroneous results in subsequent samples and can damage the analyzer. Specimen transport, storage and stability must be considered. Heat and vibration will damage hematology specimens.

3. Internal Quality Control:
   a. Establishing local QC ranges: each site must calculate its own mean and standard deviations based on parallel testing. Frequently the control material stability will be too short as to allow for performing the recommended number of runs (20); in these cases laboratories may choose to use fewer runs to calculate the mean and use historical standard deviations to put the lot into use sooner. As more runs are performed, the statistics can be updated.
   b. Levels Required: laboratories must run at least a normal and a high abnormal level. A low abnormal level may also be used.
   c. Frequency: 2 levels must be run and satisfactory by multi rule QC each day the analyzer is used. Another control should be run each additional 8 hours of testing on that day.

4. External Quality Control: HPTN/MTN sites must perform the appropriate CAP basic hematology and linearity panels. Additional external QC may be performed as desired.

5. Calibration: instrument calibration must be verified at least every 6 months, if different reagents are used or when indicated by instrument performance. If using more than one Hematology instrument, these should be compared periodically to monitor instrument to instrument agreement.

6. Specimen Analysis: results should be reviewed to confirm mathematical agreement between Hemoglobin and Hematocrit. High Mean Corpuscular Hemoglobin and High Mean Corpuscular Hemoglobin Concentrations can also indicate a problem. Any instrument generated error flags must be evaluated. All results should be reviewed by a supervisor or designee before results release.

7. Microscopy: Criteria for smear review/manual differential must be established at each site. Only technicians with adequate training should perform microscopy. Slides with suspected blasts or other extremely abnormal conditions should be reviewed by a pathologist when possible.

8. Troubleshooting specimens:
    a. NRBC: Nucleated red blood cells will be read as lymphocytes by most analyzers. The white blood cell count from samples with high NRBC should be corrected and a manual differential performed.
    b. Lipemic samples: plasma from lipemic specimens will have a milky appearance. In some case a plasma replacement may be required for these specimens.
    c. Cold Agglutinins: cold agglutinins can cause problems with Red Blood Cell indices and agreement between Hemoglobin and Hematocrit. A pre-warm procedure will frequently correct this problem.

9. Maintenance: most hematology analyzers require a shutdown cycle at least every 24 hours. Background checks are usually required after start up, reagent change and most maintenance procedures to ensure that debris is not affecting results. Follow all manufacturer recommendations.

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ROUTINE OPERATION of BECKMAN-COULTER AcT diff 2

Effective Date: 5Feb03	**Revision** #1.0	**Supercedes** AcT Manual

Prepared By	Signature	Date
Constance Ducar		

Approved By	Signature	Date
Lab Supervisor		
Lab System Administrator		
Lab Director		

Reviewed By	Signature	Date

Principle

The Beckman-Coulter AcT diff 2 analyzer is a quantitative, automated hematology analyzer and leukocyte differential counter for *in-vitro* diagnostic use in clinical laboratories. The Coulter method accurately counts and sizes cells by detecting and measuring changes in electrical resistance when a particle (such as a cell) in a conductive liquid passes through a small aperture. As each cell goes through the aperture, it impedes the current and causes a measurable pulse. The number of pulses signals the number of particles. The height of each pulse is proportional to the volume of that particle.

The AcT diff 2 analyzer uses triplicate counting, internal voting criteria and proprietary flagging algorithms to maximize the accuracy of results and confirm parameter results prior to reporting. After correction for coincidence, the three counts each for WBC, RBC and Plt are compared. If disagreement is found among count periods or other internal criteria are not met, the instrument displays and prints ------ to indicate total vote-out.

The total White Blood Cell (WBC), Red Blood Cell (RBC) and Platelet (Plt) counts are measured directly from pulses. The hemoglobin (Hgb) concentration is measured by absorbance at 525 nm against a reference blank. The remaining parameters are computed or derived as follows:

Computed: Hct, MCH, MCHC, LY#, GR#, MO#
Derived from RBC histogram: MCV and RDW
Derived from Plt histogram: MPV, Pct and PDW
Derived from WBC histogram: LY%, MO%, GR%

Specimen Collection and Handling

1. The required sample is EDTA (K_2, K_3 salt) whole blood.
2. The blood proportion to anticoagulant should be as specified by the tube manufacturer.
3. EDTA vacutainer tubes (3-7 mL) with total sample volume of < 1 mL will be noted as 'short sample'.
4. EDTA vacutainer tubes (3-7 mL) with total sample volume of < 0.5 mL should not be used due to possible anticoagulant dilution effect on the results.
5. Minimum sample size is 20 uL from a well-mixed EDTA whole blood sample of adequate total volume.
6. Samples should be stored/analyzed at room temperature (16 to 35 ^{0}C).
7. Samples should be analyzed within 24 hours of collection.
   7.1 Samples > 24 hours old but <48 hours old may be analyzed if sample replacement is not possible. Note "sample x hours old" under sample comments.
8. Do not refrigerate samples for platelet and differential counts.
9. If necessary, samples should be brought to room temperature (16 to 35 ^{0}C) before analyzing.
10. Unacceptable samples
    10.1 Clotted samples
    10.2 Samples with 3-4+ hemolysis
    10.3 Samples >48 hours old

11. Document unacceptable sample under sample comments on requisition and on computer report. Notify study/clinic as soon as possible so that the sample may be replaced in a timely manner.

Materials Required

Beckman-Coulter Diff AcT Pak reagent
Beckman-Coulter AcT Rinse Diluent
Beckman-Coulter 4C plus cell controls (low, normal and high)
Beckman-Coulter S-Cal (for calibration)
12x75 test tube or equivalent for dilutions
Normal saline (0.85-0.9% NaCl) for dilutions

Equipment Required

Beckman Coulter AcT diff 2 analyzer – ambient operating temperature of 16 to 35 $^{\circ}$C.

Precision pipette (for dilution of samples beyond linear limits)

Reagent Preparation

1. No reagent preparation is required for the Act Pak, AcT Rinse or 4C controls.
2. Refer to 'Changing Reagents' SOP for further instructions.

Storage and Stability

1. AcT Pak is stored at 2-30 ^{0}C. Unopened stability is per expiration dating listed on the Pak. Opened stability is 60 days at 16 to 25 ^{0}C and 30 days at 26 - 35 ^{0}C.
2. AcT Rinse is stored at 2- 25 ^{0}C. Unopened stability is per expiration dating listed on the bottle. Opened stability is 1 month (30 days) at 16-35 ^{0}C.
3. 4C Plus cell control is stored at 2- 8 ^{0}C and is stable per the expiration dating listed on the vials.

Calibration

1. Calibration is performed under the following conditions:
   1.1 Every six (6) months
   1.2 Whenever any instrument component involving primary measurement characteristics (such as an aperture) is replaced.
   1.3 Whenever the quality control program results suggest a calibration problem.
2. Refer to the Calibration SOP for calibration instructions.

Quality Control - Internal

1. 4C plus cell controls (low, normal, high) or equivalent will be run daily (each day of use).
2. Controls must be run prior to running any patient samples.

3. Make sure the controls are run in the correct level.
   3.1 If a control is run in the wrong level, delete the result and make a note in the corrective action log.
   3.2 If the control result is not deleted before running the next sample it will become part of the statistical database for the lot.
4. All control values must be within the expected ranges.
5. If any control value is outside the expected range, repeat the control once.
   5.1 If the control value comes in on repeat, note it on the corrective action log and continue.
   5.2 If the control value does not come in on the repeat, consult the laboratory supervisor (LS) or laboratory system administrator (LSA) for further action.
6. Review the Levey-Jennings graphs for potential QC problems. Use the Westgard Multi-rule technique as outlined in the appendix along with the data patterns presented on the Levey-Jennings graphs as indicators of possible problems. Notify the LS/LSA immediately if any of the following QC problems are noted or suspected:
   6.1 Trends
   6.2 Shifts
   6.3 Bias
   6.4 Precision
   6.5 Control degradation
   6.6 Excessive notes in the corrective action log regarding instrument or control performance (i.e. more than 1 entry per week or more than 1 entry for the same problem in one month).

7. If the instrument-reagent-control system appears to be running as expected and the controls are running within 2SD, the Levey-Jennings graphs may be printed and reviewed with the laboratory supervisor (LS) or laboratory system administrator (LSA) on Tuesday and Fridays. If problems are suspected, print the L-J's charts daily for review until resolved.
8. All unexpected results must be documented on the corrective action log along with action taken and final resolution.
9. Refer to the 'Act diff 2 4C Plus Cell Control' SOP for information on changing control lots.

Quality Control – IQAP

1. Beckman-Coulter IQAP program provides peer comparison for the 4C Plus Cell Control results.
2. At the end of each 4C lot, the lab data will be submitted to the IQAP program for additional analysis and peer comparison.
3. The LS/LSA will review and sign the IQAP reports. Any potential problems will be addressed in a timely manner.
4. The IQAP reports will be kept as part of the CoreLab External QC program.
5. Refer to the 'Act diff 2 4C Plus Cell Control' SOP for information on submission of results to IQAP.

Quality Control – Closed Vial vs. Open Vial mode

1. At least twice per year compare the closed vial to the open vial-sampling mode to ensure that both modes are functioning properly.
   1.1 Following service/calibration, check the closed vs. open vial mode.
   1.2 Check the closed vs. open vial mode any time a sampling problem is suspected
2. Perform the comparison on 3 random samples that have been collected in tubes that allow both open and closed mode sampling.
   2.1 Use the guidelines established for sample comparison in the blind/split/duplicate sample SOP for evaluating the reproducibility between modes.
   2.2 If the samples are not within the guidelines, review the results with the laboratory supervisor (LS) or laboratory system administrator (LSA).
3. File the mode comparison results in the AcT records file.

Procedure – Start-Up

1. When the instrument is turned on, it automatically performs the startup procedure.
2. If you want to run the startup procedure again, at the main screen, touch the **Startup** icon.
3. When the startup procedure is finished, the instrument will print the startup report. The report will indicate PASS or FAIL for WBC, RBC, Hgb and Plt parameters along with the background reagent count for each.
4. If the startup passes, record the start up and background counts on the daily operational check log and continue with running controls/patients.
5. If any of the parameters fail the startup and background count, repeat the startup again. Make a note under comments 'startup run X times' on the daily operational check log.
   5.1 If the startup is not passing, the instrument may need cleaned or the reagent may be contaminated.
   5.2 Do not run the startup more than 3 times without consulting the LS/LSA.
   5.3 If the background counts appear to be rising for one or more parameters or the background count is repeatedly above the guidelines, consult with the LS/LSA immediately.
   5.4 Do not run controls or patient samples until the background counts are within acceptable limits and the startup passes.
   5.5 Document any background problems (other than the occasional repeat which can be noted on the daily log) and action taken on the corrective action log.

6. Background limits:

   WBC $\leq$ 0.4
   RBC $\leq$ 0.04
   HGB $\leq$ 0.2
   PLT $\leq$ 7

Procedure – Running Controls

1. Make sure you are using the current lot of controls (ie the control values have been entered in the AcT).
2. If this is a new lot of controls, refer to the 'Act diff 2 4C Plus Cell Control' SOP for entering new lots.
3. Check that the 4C Plus cell control is not past its expiration date.
4. Remove 4C controls from the refrigerator and warm at ambient temperature for 10-15 minutes.
   4.1 Run and return the controls to the refrigerator within 30 minutes.
   4.2 Do not leave at ambient temperature for extended periods of time as this may lead to gradual deterioration of the controls over the expected shelf life.
5. Mix the controls gently by hand. Do NOT use a mechanical mixer.
   5.1 Roll the tube slowly between the palms of the hands eight times in an upright position.
   5.2 Invent the tube and slowly roll it between the palms eight times.
   5.3 Gently invert the tube eight times.
   5.4 Inspect the tube contents to determine if all cells have been uniformly distributed.
   5.5 Repeat above steps as needed until the contents have been uniformly distributed.
6. At the Main screen, touch the QA icon.
7. At the QA screen, touch the 4C Plus run icon.
8. Select the correct control level (L-low, N-normal or H-high). Make sure that the level of control you are testing matches the icon selected.
9. Invert the tube once or twice prior to cycling.
10. Place the well-mixed control tube in the tube holder at the Cap Pierce Station and close the door.
11. When the tube holder door opens, remove the vial and return it to the refrigerator.
12. Results appear on the screen.
    12.1 Check the results immediately.
    12.2 If the results are acceptable, auto-print or manually print the results for reference.
    12.3 If there is a gross error such as running in the wrong control level or obvious mis-sampling, reject the results by touching the trash icon.
    12.4 If the results are not within the expected range, rerun the control and follow the instructions in the internal QC section above.
13. When ready, run the second and third control.
14. Any unexpected results or problems must be documented on the AcT corrective action log.
15. Save 4C QC daily printouts until the 4C lot is complete and the QC records have been retrieved/reviewed from the AcT diff 2.
16. Refer to the Beckman-Coulter Operators Guide, section 2.2 Running Controls, pages 2-8 through 2-12 for diagrams of the above instructions or additional information as needed.

Procedure – Running Patient Samples – Closed Vial Whole Blood mode (CVWB)

1. Run only 4-5mL EDTA vacutainer tubes in the closed vial mode.
2. At the Main screen, select **Closed Vial Whole Blood** mode.
3. At the Main screen, touch the **Sample Results Screen** icon.
4. Check the Patient Range icon is set for 0 range (instrument's linearity limit)
5. Manually enter the lab accession number (6 numeric characters) and verify the sample ID is correct.
6. Mix the sample by hand or automatic mixer.
7. Check that you are in **Closed Vial Whole Blood** mode.
8. Place the well-mixed sample in the tube holder at the Cap Pierce Station and close the door.
9. When the tube holder door opens, remove the tube.
10. The instrument automatically saves sample results and the results appear on the screen.
11. Print the results by either Auto-print or touch the **Print** icon.
12. Refer to the Beckman-coulter Operator's Guide, section 3.2, pages 3.2-3.6 for diagrams of the above instructions or additional information as needed.

Procedure – Running Patient Samples – Open Vial Whole Blood mode (OVWB)

1. Run all samples other than 4-5mL EDTA vacutainer tubes in the open vial mode.
2. Use eye protection when running in the open vial whole blood mode.
3. At the Main screen, select **Open Vial Whole Blood** mode.
4. At the Main screen, touch the **Sample Results Screen** icon.
5. Check the Patient Range icon is set for 0 range (instrument's linearity limit)
6. Manually enter the lab accession number (6 numeric characters) and verify the sample ID is correct.
7. Mix the sample by hand or automatic mixer then remove the cap.
8. Check that you are in **Open Vial Whole Blood** mode.
9. Present the well-mixed sample in the probe so that the tip is well into the tube; press the aspirate switch.
10. When you hear the beep, remove the sample, and put the cap back on the tube.
11. The instrument automatically saves sample results and the results appear on the screen.
12. Print the results by either Auto-print or touch the **Print** icon.
13. Refer to the Beckman-Coulter Operator's Guide, section 3.2, pages 3.7-3.10 for diagrams of the above instructions or additional information as needed.

Procedure – Reviewing Sample Results

1. Check the accession number on the report against the accession number on the sample to verify it was entered correctly on the report. Add the check-digit on the sample accession number to the report.
2. Review the sample printout for possible problems.
   2.1 Critical values

2.2 Linear limits
2.3 Replacement flags
2.4 Non-Replacement flags
2.5 Clot check
2.6 Manual blood film review
2.7 Manual differential required
2.8 MCHC limits

3. If no possible problems are detected, initial and release the CBC to senior staff for second review.
4. If a possible problem is detected, take appropriate action to confirm. Document any follow-up action and results on the CBC worksheet.
5. Critical Values
   5.1 Critical values for hematology parameters are posted and are covered in detail in the critical value SOP.
   5.2 Repeat any sample that appears to have a critical value to verify the result.
   5.3 Follow the guidelines in the critical value SOP for verification of values and immediate delivery of results.
   5.4 Quick critical value reference is listed in the appendix.
6. Linear Limits
   6.1 The linear limits are summarized in the appendix.
   6.2 Samples that exceed the upper linear limits must be diluted using normal saline (0.85% NaCl) and repeated. Refer to the linear limits section in this SOP for details.
   6.3 Report printing has been set to '0 range' which prints the linear limits on the sample result report for quick reference.
   6.4 Since the operating limits for some of the parameters are broader than the linear limits, values beyond the linear limit level may print. Values beyond linear limits will have a '+' flag printed next to the result.
7. Replacement flags/codes
   7.1 These flags replace the parameter result
   7.2 Flag '-----' indicates total vote out
   Two of the three count periods did not agree or the first count period for WBC/RBC votes out.
   Thoroughly mix and rerun the sample
   If the vote out repeats, zap the apertures (see unexpected results below).
   7.3 Flag "+++++' indicates results exceed operating range.
   For WBC, RBC, Hgb or Plt, make a 1:2 dilution and repeat.
   For MCV, make a blood film and review. See also MCV section in Manual Blood Film below. Refer to linear limits section for more information.
   7.4 Flag "xxxxx" indicates aperture alert.
   Use a wooden applicator stick t check for fibrin strands or clots.
   If not clots or strands are detected, thoroughly mix and rerun the sample.
   If aperture alert repeats, zap apertures and/or clean the baths (see unexpected results below).
   7.5 Flag"….." indicates result cannot be calculated.
   Resolve parameter needed for calculation and continue.

7.6 Refer to Beckman-Coulter Operators Guide, Parameter Codes and Flags, sections 6.9-10, pages 6-80 through 6-87 for more information and diagrams.

8. Non-replacement flags/codes
   8.1 These flags appear next to the parameter results.
   8.2 Flag "+" indicates value is greater than the linear range but less than the operating range.
   Follow procedure for linear limits.
   Cycle diluent blank before proceeding with subsequent samples.
   8.3 Flag "1,2,3,4,M" indicates differential parameters failed the internal regional size distributional criteria at one (1,2,3,4) or multiple (M) regions.
   Review histogram and repeat sample if necessary.
   8.4 Flag "H" indicates a high result.
   For control samples the result is higher than the upper limit for that control sample.
   For patient samples, the result is higher than the high patient sample limit (currently not used on lab print-out)
   8.5 Flag "L" indicates a low result.
   For control samples the result is lower than the lower limit for that control sample.
   For patient samples, the result is lower than the low patient sample limit (currently not used on lab print-out)
   8.6 Flag "x" indicates one of the multiple aperture alert criteria was not met.
   Thoroughly mix and rerun the sample.
   If persist, zap the aperture.
   8.7 Refer to Beckman-Coulter Operators Guide, Parameter Codes and Flags, section 6.9-10, pages 6-87 through 6-88for more information and diagrams.

9. Clot check
   9.1 Check the sample for clots if results for multiple parameters look depressed.
10. Manual Blood film review
   10.1 If MCV is >125fL, exclude pseudomacrocytosis due to micro clots or rouleaux.
   10.2 If MCV is <55fL, exclude schistocytes, helmet cells. Confirm extreme microcytosis.
   10.3 If WBC count is >30,000 cells/uL, exclude platelet agglutination, giant platelets, nucleated erythrocytes or megakaryocytes.
   10.4 If MCHC>36.5 g/dL, check for spherocytosis. See MCHC limits.
   10.5 If MCHC <31.0 g/dL, check for low MCV/severe iron deficiency anemia. See MCHC limits.
   10.6 If platelet value is <50,000 or abnormalities are suspected from the histograms.
11. Manual differential
   11.1 If the automated differential votes out, perform a manual differential. Calculate the absolute differential values (see calculations section below). Enter the % and absolute values from the manual differential.

11.2 If the % monocytes are greater than 15, perform a manual differential. If the manual differential does not compare to the automated differential with the ranges given by a Rumke chart, report the manual differential under Diff 2. Note: occasionally high numbers of eosinophils can falsely elevate the monocyte count on the automated differentials.

12. MCHC limits
    12.1 Since MCHC varies over a narrow range, use the MCHC parameter to detect random errors, instrument malfunction or spurious results.
    12.2 If MCHC is >36.5g/dL, check blood film for spherocytosis. If spherocytosis is not present, review the results with the LS/LSA for possible error.
    12.3 If MCHC is < 31.0 g/dL, check for low MCV/severe iron deficiency anemia. If low MCV/anemia is not present, review the results with the LS/LSA for possible error.
13. Cold agglutinins
    13.1 If MCV is >125fL and manual blood film does not support macrocytosis, micro clots or rouleaux, warm tube at 36$\pm$ 2^0C for 15 minutes and re-run the sample.
    13.2 If the MCV is normal after warming, report 'possible cold agglutinin' under sample comments and report the warmed CBC hemogram.

Reporting Results

1. After second review (dated and initialed), the results are given to data entry for double entry into the computer system.
2. Report CBC results as listed on the report with any report comments that are indicated.
3. Do not report bench comments (tech notes on sample) that are intended for lab reference only.

Procedure – Shut-Down

1. Before turning off the instrument at the end of the day, perform shutdown.
2. At the Main screen, touch the **Shutdown** icon.
3. When the 'hourglass' icon/screen appears, turn off the instrument.
4. Note shutdown on the daily operational log.

Calculations – dilutions, nRBCs, corrections

1. Calculations are required under the following conditions:
   1.1 Linear limit is exceeded and a sample dilution is required
   1.2 The automatic differential voted out and a manual differential was performed – calculate the absolute count.
   1.3 Nucleated erythrocytes >8 per 100 WBC's; correct WBC count and recalculate granulocyte, lymphocyte and monocyte absolute count.
   1.4 MCV was above or below the operating range resulting in no values reported for Hct or MCHC.

2. Dilutions (linear limit exceeded)

2.1 Make a 1:2 dilution with a precision pipette using 1 part well mixed sample plus 1 part normal saline (0.85-0.9% NaCl) in a 12x75 tube (or similar container). Sample and saline volume of 100 uL is adequate.
2.2 Run the diluted sample in open mode.
2.3 Check the diluted results by comparing one of the parameters that was not above linear limits to its diluted final result.
  2.3.1 The results should be comparable.
  2.3.2 If not the dilution may be erroneous. Remake the dilution and run again.
2.4 Correct the diluted result by multiplying by 2 (dilution factor). Report the corrected diluted result along with the other undiluted parameters that are within linear limits.
2.5 Do not report corrected diluted results for parameters that are within linear limits.
2.6 Example:

Parameter	Undiluted	Diluted x2	Report
RBC	7.2	3.7	7.4 (3.7 x 2)
Hgb	20.8	10.8	20.8
HCT	59.6	21.2	59.6
Plt	214	110	214

Note agreement between diluted x2 and undiluted parameters for Hgb, HCT and Plt verifying dilution accuracy. Note only RBC value has been corrected since the remaining parameters were within linear limits on undiluted sample.

3. Calculation of absolute leukocytes per category:
3.1 Absolute count (10^3 cells/uL) = (%count/100) x WBC count
3.2 Example:
WBC = 7.8 x 10^3 cells/uL
% lymphocytes = 48%

Absolute Lymphocyte Count (Ly#) = (48/100) x 7.8 = 3.744 x 10^3 cells/uL

4. Correction for >8 nucleated erythrocytes (nRBCs) per 100 WBC's
(refer to Nucleated Erythrocytes SOP for details)

4.1 Total WBC count correction

$$\frac{\textbf{Uncorrected total WBC count}}{100 + \#\text{nRBCs per 100 WBC's}} \textbf{ X 100}$$

Example: 23.8 x 10^3 cells/uL

$$\frac{\text{---------- X 100}}{100 + 9\text{nRBC's per 100 WBC's}} = 21.8 \times 10^3 \text{ cells/uL}$$

4.2 Calculation of absolute WBC counts by category

(% count/100) X corrected WBC count

Example: corrected WBC count = 21.8 x 10^3 cells/uL
% lymphocytes (or granulocytes or monocytes) = 45%

(45/100) x 21.8 = 9.81 x 10^3 cells/uL

5. MCV <50 or >130 fL (no Hct or MCHC results)
   5.1 Check slide first to establish validity of low or high MCV. If MCV result is not valid, refer to possible problems in result review section above. Do not calculate HCT or MCHC unless low/high MCV appears valid.
   5.2 Calculate HCT as an approximation of the Hgb using the rule of three. (rbcx3 ~hgb, hgbx3 ~ hct)

   Hgb x 3 ~ HCT, if Hgb = 8.4 then HCT is approximately 25.2
   Report HCT as approximately 25.2

   5.3 Calculate MCHC as if HCT = 25.2%

   MCHC (g/dL) = Hgb/Hct x 100 → 8.4 / 25.2 x 100 = 33.3
   Report MCHC as approximately 33.3

   5.4 Report MCV as < 50 (or >130) with comment 'verified by blood film'

Unexpected Results

1. If high background counts persist, try zapping the apertures or cleaning the baths. Refer to the Maintenance SOP for details.
2. If unexpected sample results are encountered,
   2.1 Repeat the sample
   2.2 Review the possible sample problem section
   2.3 Check the QC records
   2.4 Check blood film if appropriate
   2.5 Review the sample results with the LS or LSA

Reference ranges

Reference ranges for the AcT have been established for the Ugandan population taking into account sex, pregnancy and age. Current reference ranges are included in this manual. (see index)

Linear Range

1. Sample results are set to print with associated range "0". Range "0" references the instruments linearity range on printouts as a reminder.
2. The linear range is the range that sample results may be directly reported. Values outside the linear range can NOT be reported without dilution and correction.
3. Do not confuse linear range with operating range. The operating range is wider than the linear range in most instances. Values will print that are outside of the linear range but within the operating range. Values outside the linear range that are within the operating range must still be diluted and repeated.
4. Refer to calculation section for dilution and calculation instructions.

Parameter	Linear Limit	Operating Limit
WBC, x 10^3 cells/uL	0 – 99.9	0 - 150
RBC, x 10^9 cells/uL	0 – 7.0	0 – 8.0
HGB, g/dL	0 – 25.0	0 – 30.0
PLT, x 10^3 cells/uL	0 – 999	0 - 3000
MCV	50 – 130	50 - 130

Limitations of the Procedure (Interfering substances)

Refer to the Interfering Substances chart in the appendix for a list of possible interfering agents and conditions.

Performance Specifications

Refer to the Beckman-Coulter Reference manual, Performance Specifications, section 4.2, pages 4-4 through 4-6 and Performance Characteristics, section 4.3, pages 4-7 through 4-11 for detailed information.

Procedural Notes

1. The background count after start-up is sometimes high if the instrument has been sitting for >24 hours such as over weekends and holidays. The high background count will clear with a few cycles. Alternatively, the instrument can be cycled through start-up/shut-down on weekends and holidays.
2. Use associated range "0" for printouts. Range "0" references the instruments linearity range on printouts as a reminder.

References

Beckman-Coulter Operator's Guide
Beckman-Coulter Reference manual
Beckman-Coulter Installation and Training Guide
Beckman-Coulter Basic Concepts of Quality Control manual

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COULTER ACT DIFF-2 DAILY OPERATIONAL CHECK LOG MONTH_____YEAR_____

Date	Clean bench	Wipe AcT 10% bleach	Operation Light on	SpleHlder Open	Beckman Logo on	Automatic Startup done	WBC <0.4	RBC <0.04	Hgb <0.2	Plt <7.0	QC Run &Rev'd	Shut Down	Comments	Tech Sign
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
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Coulter AcT diff 2 Record and Report Summary

Coulter AcT diff 2 Logs

1. Operational Log
2. Reagent Log
3. Corrective Action Log
4. Calibration Log

Calibration

For each calibration save the following records:

1. Print-out of the current calibration factors.
2. Print-out of the reproducibility check summary
3. Print-out of the carry-over summary
4. Print-out of the calibration summary
5. Print-out of the new calibration factors.
6. S-Cal insert showing the constituent values used for the calibration.
7. Calibration records will be reviewed and signed by the lab supervisor.

In addition:

1. Record new calibration factors on the calibration log sheet along with the reagent information.
2. Run all 3 levels of 4 C post calibration to verify the calibration.
3. Place calibration sticker with the current calibration date and the next re-calibration dateon the Coulter AcT diff 2.

Daily 4C QC

1. Save the background and 4C printouts from each day until the 4C lot file is closed and the summary/Levy Jennings charts are printed.
2. All 3 levels of 4C will be run once daily.
3. If all controls pass proceed with the patient samples.
4. If the control is run in the wrong QC file (ie normal control run in the low file etc), delete the results and make a note in the Corrective Action Log.
5. If any of the controls are outside the lot specific ranges, rerun that control before proceeding with patient samples.
6. If the repeat control passes, document the problem and correction on the corrective action log. Proceed with patient samples.
7. If the repeat control does not pass, consult the lab supervisor. Patient samples can not be run until the supervisor has resolved the problem. The problem and action taken must be documented on the corrective action log.

4C Lot Change

1. Download the QC records onto the reagent card for submission to IQAP.
2. Print the QC Summary and the Levy-Jenning charts.
3. Review and file the summary, charts and 4C lot sheet. Add the IQAP report when received.
4. Clear (delete) the old QC records from the summary and chart files.
5. Enter the new 4C values for each level.
6. Print the new values and check against the 4C lot record.

HPTN Central Lab Reports (send to Estelle Piwowar-Manning at the end of each lot of 4C.)

1. QC Summary report for each level
2. L-J charts for each level
3. Operational Log(s) for the time period of the 4C lot
4. Corrective Action log for the time period of the 4C lot

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AcT diff 2 Range and Review Limits

Parameter	Critical Value	Linear Limit	Operating Limit	Review Limit	Comments
WBC, $x10^3$ cells/uL	<1.5	0 – 99.9	0 - 150		
WBC, $x10^3$ cells/uL	>30.0	0 – 99.9	0 - 150	>30,000	Blood film review
RBC, $x10^9$ cells/uL	<2.4	0 – 7.0	0 – 8.0		
Hgb, g/dL	<5.5	0 – 25.0	0 – 30.0		
Hct, %	<16				
Plt, $x10^3$ cells/uL	<50	0 - 999	0 - 3000	<50	Blood film review
MCV, fL		50 - 130	50 - 130	<55 or >125	Blood film review
MCHC				<31.5 or >36.5	Blood film review
Differential, %			0-100%	Vote-Out	Manual Differential
Diff, $x10^3$ cells/uL			0 – 99.9	Vote-Out	Manual Differential
% monocytes				>15%	Manual Differential

Procedure: **COULTER T540 MONTHLY REPRODUCIBILITY CHECK**

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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PRINCIPLE:
Monthly reproducibility checks will be run to verify the T540 is performing within acceptable guidelines.

MATERIALS REQUIRED:
Normal EDTA blood sample
Uni-T Pak

EQUIPMENT REQUIRED:
Coulter T540
Sample rocker/mixer

STORAGE/STABILITY: The normal blood sample should be less than 6 hours old and stored at room temperature.

PROCEDURE:

1. Perform routine start-up and daily control run.
2. Check that there is adequate reagent for 21 samples before starting.
3. Select a 'normal' blood sample with adequate volume for 21 tests that is less than 6 hours old.
4. Mix the sample well.
5. Run the sample to prime the T540 (sample 0). Do not include this sample in the reproducibility calculations.
6. Run the sample 10 times taking care to mix well between each sampling. Do not run any other samples in between the reproducibility check samples.
7. Follow the instructions under calculations to determine the reproducibility. Document the results on the Reproducibility Check Log.
8. If the results are within the expected guidelines, sign the log and file the results in the bench log.
9. If the results are outside the expected guidelines, repeat the reproducibility check a second time taking care to mix very well between samples.
10. If the second run passes, sign this log with a note "second run". File both the first and the second run results in the bench log.
11. If the second run fails, review results with the LS (Laboratory Supervisor).

CALCULATIONS:

1. Use the Reproducibility Check Log to calculate the differences.
2. For each parameter:
   2.1 Find the high and low value out of the 10 samples.
   2.2 Subtract the two to obtain the difference
   2.3 Compare the differences to the acceptable range listed on the log.
3. The high-low difference for each parameter should fall within the acceptable range listed for that parameter.

INTERPRETATION OF RESULTS

1. If the results are within the guidelines listed, the T540 is performing within the expected guidelines for reproducibility.
2. Results not within the guidelines may indicate a problem with the T540 and should be followed up immediately.

LIMITATIONS OF THE PROCEDURE

1. The reproducibility guidelines are for samples with normal values.
2. Problems may be encountered if the sample is not normal or the sample integrity has been compromised (sample age, exposure to temperatures higher than room temperature for extended periods of time.

REFERENCES:

1. Operators Handbook for the Coulter Counter Model T Series Instruments, Dec 1989.
2. Reference Manual for the Coulter Counter Model T Series Instruments, Dec 1989.

APPENDIX:

1. Reproducibility Check Log

Procedure: NUCLEATED ERYTHROCYTES (nRBC)

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducor		

Review Date	Revision Date	Signature

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Principle: The presence of nucleated erythrocytes in the peripheral blood is an indication of increased erythropoietic activity within the bone marrow or infiltration of the bone marrow. It is most frequently observed in the blood of newborn babies.

The Coulter T540 will read nucleated erythrocytes as small lymphocytes. If the number of nucleated erythrocytes (RBCs) is high, the total white blood count, the absolute lymphocyte count and the percentage lymphocytes will be falsely elevated. When elevated numbers of nucleated erythrocytes are observed by a manual differential cell count, the white blood parameters must be corrected.

Equipment Required – Calculator

2.Procedure

1. When performing a manual differential, note the number of nucleated erythrocytes (nRBCs) seen per 100 white blood cells (WBCs).

1.1 If an occasional nRBC is seen while scanning for morphology, note "Rare nRBC seen" under comments
1.2 If one or more nRBCs are seen while counting 100 WBCs, note "# nRBCs/100WBCs seen" under comments.
1.3 If rare to 8 nRBCs are noted no correction for the WBC parameters are required.
1.4 If more than 8 nRBCs per 100 WBC's are seen, the WBC parameters must be corrected.

2. If more than 8 nRBCs per 100 WBCs are seen on the manual differential, perform the following calculations to correct the WBC parameters.

2.1 Corrected WBC count

$$\frac{\text{Uncorrected total WBC count}}{100 + \#\text{nRBCs per 100 WBCs}} \times 100$$

2.2 Corrected Absolute Lymphocyte

$$\frac{\text{Corrected WBC count}}{\text{Uncorrected WBC count}} \times \text{Uncorrected Absolute Lymphocyte Count}$$

2.2 Corrected Percentage Lymphocyte

$$\frac{\text{Corrected Absolute Lymphocyte Count}}{\text{Corrected WBC Count}} \times 100$$

Example: WBC 25,500
AbsLymph 4.2 x 10^3
% Lymph 16.5
nRBC 14/100 WBC

$$\text{Corrected WBC Count:} \quad \frac{25.5}{100 + 14} \times 100 = 22.4 \times 10^3$$

$$\text{Corrected AbsLymph:} \quad \frac{22.4}{} \times 4.2 = 3.7 \times 10^3$$

25.5

$$\text{Corrected \%Lymph:} \quad \frac{3.7}{22.4} \times 100 = 16.5\%$$

3. Changes in the absolute lymphocyte value will affect the calculation of %CD4 and %CD8.
   3.1 If the absolute lymphocyte value is changed, check to see if a CD4 or CD4/CD8 were also ordered on this specimen.
   3.2 If there is a CD4/CD8 on this specimen, notify the technologist performing the CD4/CD8 of the revised value.
   3.3 If the CD4/CD8 on this specimen has already been reported:
      3.3.1 Recalculate the %CD4 and %CD8 using the corrected absolute lymphocyte value.
      3.3.2 Mark the correction on the CD4/CD8 bench log and initial.
      3.3.3 Give to the Group Lead or Lab System Administrator for an amended report.

References

1.Clinical Diagnosis and Management by Laboratory Methods, Henry, 19th Edition,"Basic Examination of Blood", Chapter 24, page 557.

Procedure: DIFFERENTIAL STAIN CHECK LOG - 2001

Prepared by	Date Adopted	Supersedes Procedure #

Review Date	Revision Date	Signature

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1. Check differential stain for acceptable color on first slide each day slides are read.
2. RBC's should be pink, WBC's should be bluish and there should be no significant background debris.
3. Re-filter the stain if there is excessive background debris.
4. Make fresh slides and re-stain if the color is off.
5. Make fresh stain and discard the old stain if the color does not correct after filtration.
6. The stain must be labeled with the date prepared and your initials.

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Comments:_____
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Section 11: Biochemistry

1. Overview: Biochemistry is a broad term which describes many quantitative and qualitative tests blood and body fluids and is an essential component of safety testing in clinical trials. Common tests performed in HPTN and MTN studies include liver function tests, renal function tests, electrolytes, cardiac function tests, lipase, magnesium, calcium, uric acid, and lipid profile. A clinical laboratory that services a general patient population will have a much more comprehensive test menu.

   Many biochemistry tests are now performed using automated analyzers. These vastly increase the testing volume that can be generated by a laboratory. Analyzers are frequently classified as open or closed. Closed systems accept only reagents from the manufacturer while open systems are more flexible and can accept reagents from other sources. Discrete analyzers allow specimens to be tested for a single analyte or a panel of tests.

2. Specimen: most chemistry tests are performed using serum. Some tests may require plasma and special handling; an example of this is ammonia. Urine and other body fluids are also commonly tested.

3. Internal Quality Control:
   a. Establishing local QC ranges: each site must calculate its own mean and standard deviations based on parallel testing. At least 20 runs are required. The new lot should be run in parallel with the old lot to ensure instrument performance while establishing the new ranges.
   b. Levels required: laboratories must run at least a normal and a high abnormal level. A low abnormal level may also be used.
   c. Frequency: 2 levels must be run and satisfactory by multi rule QC each day the analyzer is used. Another control should be run each additional 8 hours of testing on that day.

4. External Quality Control: HPTN/MTN sites must perform the appropriate CAP basic chemistry and linearity panels. Additional external QC may be performed as desired.

5. Calibration: as opposed to Hematology instruments which are generally calibrated every 6 months, chemistry analyzers will need to be calibrated more frequently. The schedule of calibration will vary for each test and frequently methods will be calibrated with each lot change or even for new cartridges.

6. Specimen Analysis: the units in which results are reported may vary from country to country and this needs be examined to avoid erroneous reporting of results. Results should be reviewed by a supervisor before release.

7. Microscopy: N/A

8. Trouble shooting Specimens:
    a. Dilutions: specimens with high values will frequently need to be diluted to perform analysis. Systems need to be in place to ensure accurate dilutions and related calculations.
    b. Lipemia: a condition where the serum or plasma has a milky appearance. This can interfere with optical measurements. In some cases it can be remedied by dilution; severe cases may require a new samples.
    c. Hemolysis: a reddish appearance caused by rupturing of red blood cells. This will effect specific tests (glucose, potassium etc…). In moderate to sever cases a new sample is required.

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9. Maintenance: automated analyzers frequently require high levels of maintenance and trained technicians who are able to trouble shoot. It is recommended that a service contract be in place for all major biochemistry equipment as many problems will require trained specialists. A maintenance plan following manufacturer's instructions must be strictly followed. Most biochemistry analyzers require Type I or other laboratory grade water. If an external water plant is used, there must be appropriate maintenance of the plant and QC of the water quality.

Roche AVL 9180 Biochemistry Procedures		
SOP Number:	Total Number of Pages: 12	
Primary Author: L. Ameedha / P.Nandagopal	Signature & Date:	
Reviewed by: M. Sundaram, E. Livant	Signature & Date:	
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Approved by: Dr.Balakrishnan, Dr. K.G. Murugavel	Signature & Date:	
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SCOPE

This SOP applies to all testing performed in YRG CARE Infectious Disease Laboratory using the Roche AVL 9180. This SOP does not replace complete training and only authorized personnel should perform testing using the AVL. Specific directions for use of the AVL 9180 can be located in the Roche 9180 User's Manual.

ROCHE AVL 9180 PRINCIPLE

The 9180-analyzer methodology is based on the ion- selective electrode (ISE) measurement principle to precisely determine the measurement values.

There are three different electrodes used in the 9180 Electrolyte Analyzer: sodium, potassium, and chloride. Each electrode has an ion-selective membrane that under goes a specific reaction with the corresponding ions contained in the sample being analyzed. The membrane is an ion exchanger, reacting to the electrical charge of the ion causing a change in the membrane potential, or measuring voltage, which is built up in the film between the sample and the membrane. All results are reported in mmol/L.

CLINICAL SIGNIFICANCE

Chloride

Chloride is an anion that exists predominantly in extra cellular spaces. It maintains cellular integrity through its influence on osmotic pressure. It is also significant in

monitoring acid-base balance and water balance. In metabolic acidosis, there is a reciprocal rise in chloride concentration when the bicarbonate concentration drops.

Decreased levels are found in severe vomiting, severe diarrhea, ulcerative colitis, pyloric obstruction, severe burns, heat exhaustion, diabetic acidosis, Addison's disease, fever and acute infections such as pneumonia.
Increased levels are found in dehydration, cushing's syndrome, hyperventilation, anemia, and cardiac decompensation.

Potassium

Potassium is the major cation in the intracellular fluid and functions as the primary buffer within the cell itself. Ninety percent of potassium is concentrated with in the cell, and damaged cells release potassium into the blood. Potassium plays an important role in nerve conduction, muscle function, and helps maintain acid-base balance and osmotic pressure.

The potassium value obtained may be used to monitor electrolyte imbalance in the diagnosis and treatment of infusion therapies, shock, heart, or circulatory insufficiency, acid-base imbalance, therapy with diuretics, kidney problems, diarrhoea and hyper-and hypo- function of adrenal cortex and other diseases involving electrolyte imbalance.

Sodium

Sodium is the major cation of extra cellular fluid. Its primary functions in the body are to chemically maintain osmotic pressure and acid- base balance and to transmit nerve impulses. Sodium functions at the cell membrane level by creating an electrical potential between different cell membranes causing the transmission of nerve impulses and neuromuscular excitability to be maintained. Sodium is involved in some enzyme-catalyzed reactions as a cofactor. The body has a strong tendency to maintain a total base content, and only slight changes are found even under pathologic conditions.

EQUIPMENT AND MATERIALS

Equipment

- 100-1000 µl adjustable pipette
- 1 ml non adjustable pipette

Materials

- Pipette tips
- Empty reagent containers
- 2 ml screw top vials
- 14 ml Falcon tubes
- Gloves
- Splatter or face shields
- 10% bleach
- Clerical supplies

QUALITY CONTROL

Each day testing is performed, two levels of Roche ISETROL control must be run before reporting patient results. ISETROL control (Roche CAT# 03112888180) comes in tri level and these should be rotated between days of usage. If any of the control results violate the current YRG CARE Infectious Disease Laboratory Multi Rule Algorithm for quantitative test results (See Appendix A), corrective action must be taken and documented before results can be released for that specific analyte. Technicians may use judgment and take corrective action on the analyzer before QC rules are violated.

Further, if the number of patient samples analyzed for any parameter exceeds 75 per day, the laboratory shall employ 2 levels of QC at least twice a day at appropriate intervals for that specific day.

Corrective action may include

- re-calibration
- electrode cleaning or reconditioning
- changing of control materials or other actions as described by Roche in the AVL 9180 user's manual.

- If the technician is unable to correct the problem, technical service must be contacted. QC failures and corrective actions should be in documented.

All QC results, including QC results which failed, must be recorded on the daily QC log sheets and reviewed daily by the technician. These results may be entered daily in the AU 400 Excel spread sheet to generate Levey-Jennings charts to facilitate interpretation of QC results and rules violations. At the very least, the results must be entered monthly and these spreadsheets should be archived and backed up on separate media. The QC results will be reviewed by monthly by a supervisor or designee. The QC results will also be sent to Johns Hopkins University monthly for review.

REAGENTS

Reagents used for the AVL 9180 include the SnapPak reagent pack (Roche CAT # 03112349180), electrode deproteininzer (Roche CAT # BP0521), electrode conditioner (Roche BP0380), and Roche ISETROL controls (in tri level).

Preparation

All reagents come ready for use and no preparation is required. Controls come in single use ampoules and may be transferred into 2 ml screw top vials after ampoules are opened to extend usage. These vials must be labeled with the control material name, lot number and expiration date.

Stability and Storage

All reagents are stored at room temperature 15°C to 30°C until the expiration date listed on the container.

Calibration

The AVL is auto-calibrates each 4 hours and after daily maintenance.

SPECIMEN TYPES AND STABILITIES

Specimens that are unacceptable for testing must be rejected. The technician should fill out a Specimen Rejection form and send it back to the appropriate health care provider.

Some specimens with issues such as mild hemolysis or lipemia may be analyzed; the patient report must include comments of the problem.

Specimens are routinely stored at room temperature after analysis until the next working day. If problems are suspected or testing cannot be completed the same day of receipt, specimens may be refrigerated or frozen for further testing. Refer to the Abnormal Results section for more information.

The specimen type is serum, either from Serum Separator (SST) or non-anticoagulated red top tube. Separate from cells immediately. Separated serum is stable at 2-8°C for 30 days and at 15-25°C for 7 days. If storage is required, the sample should be stored, tightly capped, under refrigeration at 4 to 8°c and allowed to return to room temperature, 15 to 30°c, prior to analysis.

Potassium is heavily affected by hemolysis; any specimen with more than slight hemolysis should not be analyzed for potassium.

MAINTENANCE

Daily Maintenance

1. From the standby screen press “YES” twice, then “NO”
2. The screen will display “Daily Maintenance?” Press “YES”.
3. The screen will display “Perform daily cleaning?” press “YES”. (This is the deproteinization step)
4. Raise the probe and introduce the cleaning solution.
5. After aspiration, remove the sample and wipe the probe.
6. Replace the probe in the machine.
7. After completion of the cleaning, the screen will display “Perform daily condition?” Press “YES”.
8. Open the probe and introduce the conditioning solution.
9. After aspiration, remove the solution and wipe the probe.
10. Replace the probe in the machine.

11. After conditioning, the machine will calibrate automatically.
12. Perform documentation.

Weekly, Monthly, Quarterly and Annual

All other scheduled maintenance is performed by Technical specialists.

Unscheduled Maintenance, QC Issues, and Troubleshooting

Any type of maintenance that is not part of scheduled service, performed either by YRG staff or by CPC service staff must also be documented. These may be as a result of QC failure, QC trends, error messages, mechanical failures, or abnormal patient results that indicate potential instrument problem. Log sheets will be kept in the chemistry department for documentation.

TESTING SAMPLES/ WORKFLOW

1. Each morning, check room temperature. It should be 25°C ± 5°C.
2. Discard the samples from the previous day.
3. Clean work area.
4. Perform and document any daily and any other maintenance as needed.
5. Run, review and document daily QC.
    a. From the main screen, press "NO" as many times as needed to get to the desired QC level.
    b. Press "YES", raise the probe and introduce the QC sample.
    c. After aspiration, remove the sample and wipe the probe.
    d. Replace the probe in the machine.
    e. Store the QC values in the machine and record them on the QC log.
    f. Perform documentation.
6. Specimens will be received first in the specimen processing unit adjacent to chemistry. The specimen processing unit will deliver the appropriate specimens and test requisition forms (CCPL) to chemistry.

7. Chemistry personnel must first check that specimens are properly labeled with necessary information. They will then check that there is a corresponding CCPL with the correct information.
8. The specimen must then be evaluated for correct type and specimen integrity. Unacceptable specimens should not be processed and a specimen rejection from must be filled out and dispatched to the clinical unit. Specimens with slight problems may be processed and documented on the CCPL at the discretion of the technician.
9. Specific Criteria for Specimen Rejection:
    a. Wrong specimen type (Example: whole blood instead of serum)
    b. Missing or incomplete Specimen ID#
    c. Greater than mild hemolysis
    d. Specimens that have not been properly transported and handled
    e. Specimens that are too old
    f. Insufficient quantity
10. Specimens requiring electrolyte analysis are tested on the AVL 9180.
11. AVL 9180 results are entered on the tracking sheet.
12. To analyze samples
    a. From the main screen press “YES” twice.
    b. Open the sample probe and introduce the patient sample.
    c. After aspiration, remove the sample and wipe the probe.
    d. Replace the probe in the machine.
    e. Record the result on the specimen tracking form.
13. From the specimen tracking form results are transcribed into the patient results register.
14. Results are transcribed to the CCPL forms. Transcription is double checked against the tracking form.
15. The patient results register is the source document for these tests unless otherwise specified for specific studies. The tracking forms are not maintained because they are potentially biohazardous.

16. Results are entered into the Hospital Management System (HMS) from the CCPL forms. The HMS generated reports are checked against the CCPL forms before release to the clinical units.

REPORTABLE RANGE (LINEARITY)

Each analyte has a defined reportable range or linear range. When a specimen's initial result is higher than the reportable range, retest the specimen. If the results agree, the specimen should be diluted and retested. The diluted result must then be multiplied by the dilution factor to get the actual result. The result obtained from the analyzer (before multiplication by dilution factor) must be within the reportable range or a higher dilution factor may be warranted.

Specific information regarding types of diluents, dilution procedures and dilution limits should be obtained from the appropriate package inserts.

In certain cases it will be impossible to generate a result through dilutions. In these cases, the result should be given as "greater than X" with X referring to the upper limit of the reportable range.

When the initial test result is less than the lower limit of the reportable range, the specimen should be retested. If the results agree the, result should be given as "less than Y" with Y referring to the lower limit of the reportable range.

In all cases, technician judgment must be used when results fall outside the reportable range. In cases where it is possible the specimen has been contaminated or other issues with specimen integrity are apparent, the specimen should be rejected.

Refer to Table 1 for a list of linearities for specific analytes. These ranges are from package inserts and are subject to change. They may be modified by local linear range validation experiments.

Table 1 Linearity

Analyte	Linearity
Chloride	73 to 137 mmol/L
Potassium	1.1 -13 mmol/L
Sodium	96 to 193 mmol/L

ABNORMAL RESULTS

1. Recheck specimen integrity (clotted specimen, hemolysis etc….)
2. If analyzer performance is in doubt, perform corrective action or rerun a control. Analyzer performance should be evaluated if many specimens yield abnormal or highly abnormal results when not expected.
3. If steps 1 and 2 are acceptable, repeat the analysis
4. If result is outside linear range, perform dilution based analysis when appropriate. See Linearity section and package inserts for more information.
5. Consult a supervisor or second technician when needed.
6. If clinical information is available, this can be used to evaluate if the result is biologically plausible.
7. When the validity of the result does not make clinical sense or is in doubt, do not report results and request a new sample. Communicate with clinician the issues.
8. Provide proper documentation as to actions taken.
9. Abnormal specimens should be retained for 30 days frozen at <-12° in case of requests for further testing.

REFERENCE RANGES

Reference ranges (also known as normal ranges or intervals) are used by clinicians to evaluate results from quantitative diagnostic laboratory tests. Laboratory technicians should also be aware of these ranges so that the can evaluate if results obtained make biological sense. Refer to Table 2 for a list of reference ranges for specific tests.

Table 2 Reference Ranges

Analyte	Reference Range
Chloride	100-107 mmol/L
Potassium	3.8-5.0 mmol/L
Sodium	135-142 mmol/L

TURN AROUND TIME

- Turn around time (TAT) for chemistry is defined as when the sample is received from the specimen processing department to when the result is given to the data clerks.
- Routine TAT is set at ≤ 3 hours.
- When specimens are received with comments indicating they are needed urgently or stat, the TAT is set at ≤ 1 hour.
- Refer to the current version of the YRG CARE SOP for critical values for special handling in cases of certain values extremely outside of normal ranges.
- Conditions which delay reporting of results should be documented and communicated to laboratory mangers and clinics as needed.

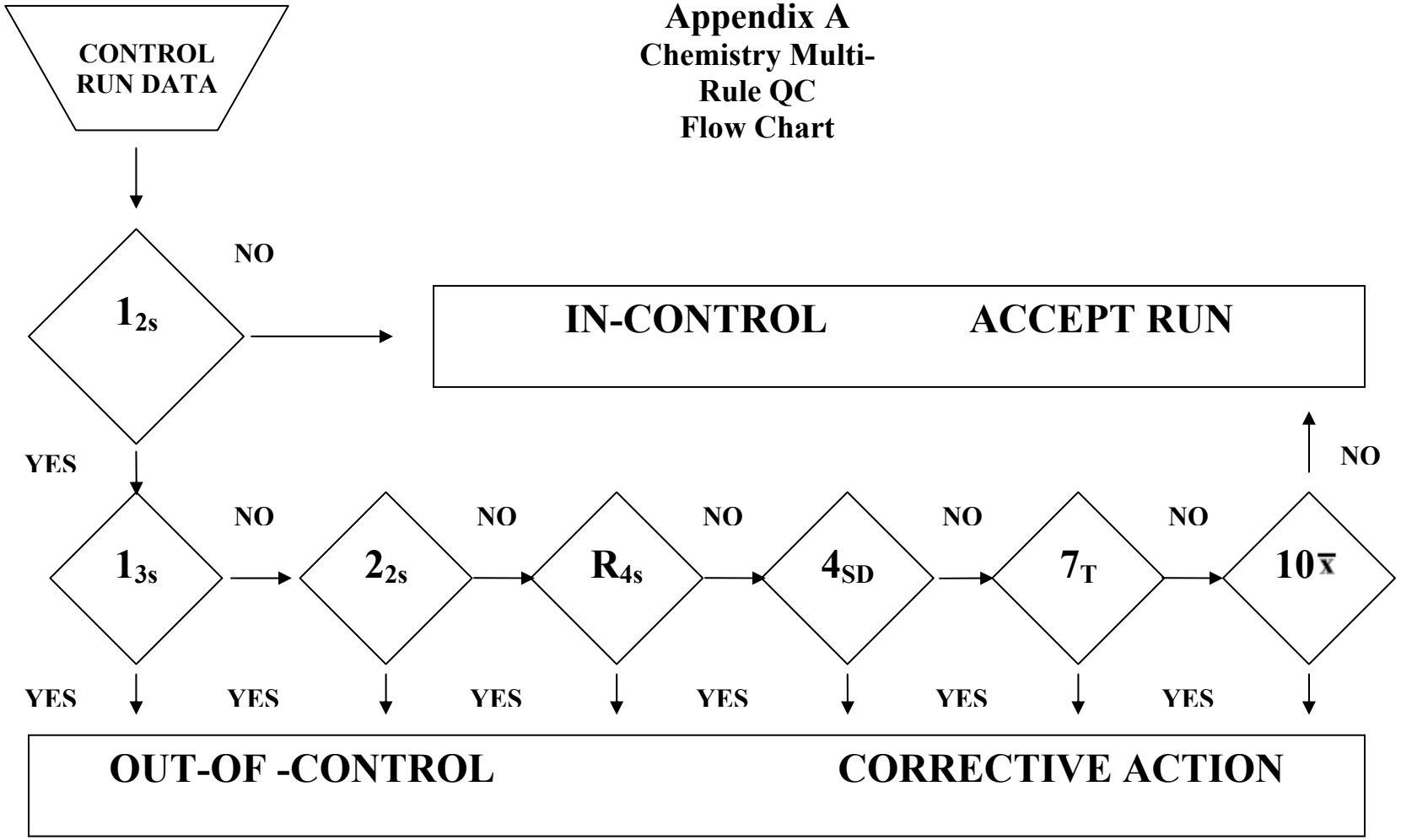
CRITICAL VALUES

Table 3 lists critical values in chemistry. Immediate action must be taken in these cases. All chemistry technicians must be aware of these values and take appropriate action as described in the version of the YRG CARE critical values SOP.

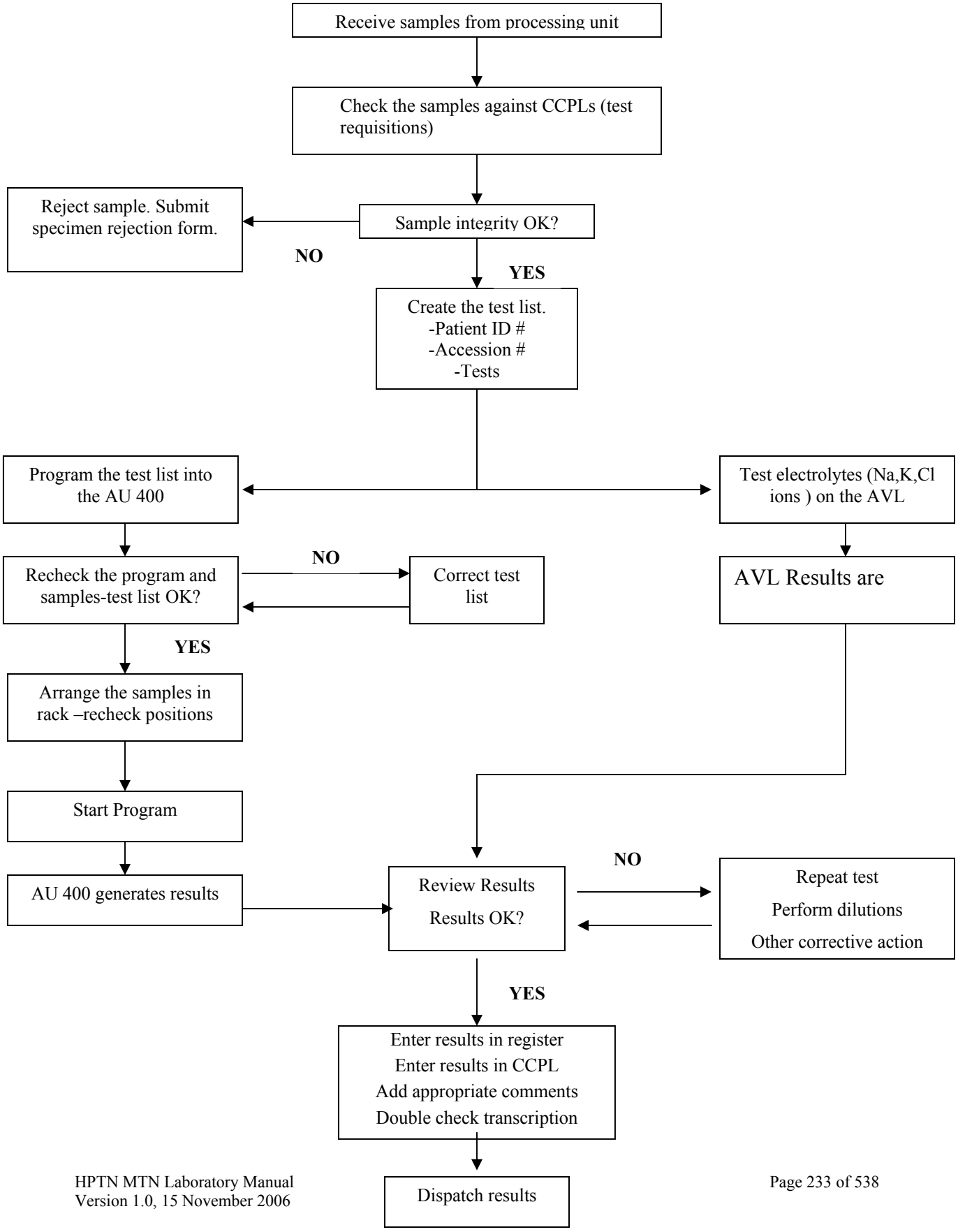
Table 3. Chemistry Critical Values

Parameter	Lower limit	Upper limit
Glucose	< 40 mg/dL	> 450 mg/dL
Potassium	< 2.8 mmol/L	> 6.2 mmol/L
Sodium	< 120 mmol/L	> 160 mmol/L
Calcium	< 6.0 mg/dL	> 13 mg / dL
Creatine Kinase	N/A	> 1000 U/L
Magnesium	< 0.9 mg/dL	> 3.7 mg/dL
Urea	N/A	> 85 mg/dL

Appendix A
Chemistry Multi-Rule QC Flow Chart



APPENDIX B: CHEMISTRY SPECIMEN PROCESSING WORKFLOW



Olympus AU 400 Biochemistry Procedures		
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SCOPE

This SOP applies to all testing performed in YRG CARE Infectious Disease Laboratory using the Olympus AU400. This SOP does not replace complete training and only authorized personnel should perform testing using the AU 400. Specific directions for use of the AU 400 can be located in the Olympus AU 400 User's Manual. Refer to SOP's for individual analytes for more information.

OLYMPUS AU 400 PRINCIPLE

This system optically measures the components of a sample (serum, urine or other samples such as CSF, Pleural fluid etc…). First a sample is dispensed, then a reagent is added to the sample. After mixing the sample with the reagent and appropriate incubation, the system measures the absorbance of the sample at specific wavelengths. This reaction may be measured once (endpoint) or there may be multiple measurements (kinetic). The absorbance measurement is used to calculate an optical density. The optical density or densities are then converted in to appropriate units such as mg/dL or IU/L.

EQUIPMENT AND MATERIALS

Equipment

- 100-1000 µl adjustable pipette
- 1 ml non adjustable pipette

Materials

- Pipette tips
- Empty reagent containers
- AU 400 accessory kit
- 2 ml screw top vials
- 14 ml Falcon tubes
- Gloves
- Splatter or face shields
- AU 400 racks
- AU 400 sample cups
- 10% bleach
- Clerical supplies

QUALITY CONTROL

Each day testing is performed, two levels of BIO RAD Lyphochek Control must be run before reporting patient results (L1 CAT# C-310-5; L2 Cat# C-315-5). If any of the control results violate the current YRG CARE Infectious Disease Laboratory Multi Rule Algorithm for quantitative test results (See Appendix A), corrective action must be taken and documented before results can be released for that specific analyte. Technicians may use judgment and take corrective action on the analyzer before QC rules are violated.

Further, if the number of patient samples analyzed for any parameter exceeds 75 per day, the laboratory shall employ 2 levels of QC at least twice a day at appropriate intervals for that specific day.

Corrective action may include

- re-calibration
- changing of reagents
- changing of control materials
- other actions as described by Olympus in the AU 400 user's manual.

- If the technician is unable to correct the problem, service engineers must be contacted and documented.

All QC results, including QC results which failed, must be recorded on the daily QC log sheets and reviewed daily by the technician. These results may be entered daily in the AU 400 Excel spread sheet to generate Levey-Jennings charts to facilitate interpretation of QC results and rules violations. At the very least, the results must be entered monthly and these spreadsheets should be archived and backed up on separate media. The QC results will be reviewed by monthly by a supervisor or designee. The QC results will also be sent to Johns Hopkins University monthly for review.

REAGENTS

Preparation

The reagents arrive from the manufacturer ready to use and require no special preparation unless otherwise noted here or in the SOP for that analyte. Ready to use test reagents are aliquoted from manufacturer's containers to analyzer on board containers based on appropriate levels for daily usage. This must be documented in the "Internal Indent Register".

Proper use of personnel protective equipment and chemical handling standards must be followed while preparing and manipulating all these reagents.

Cuvette Wash Solutions

Base wash solution: Dilute 5% sodium hypchlorite solution 1:10 with deionized water.
Acid wash solution: 1 Normal HCl comes ready for use from manufacturer.

W1 Solution

Prepare a 10% dilution of Olympus Extran (CAT # 60755050001046) solution with deionized water.

BIORAD Lyphochek Controls and Olympus System Calibrator

Spread a clean sheet of filter paper on the working table before starting the procedure. The entire procedure is carried out in a clean working table with filter paper to prevent any contamination of the QC materials or calibrators.

Using a volumetric pipette, reconstitute each vial with 5 ml of deionized water. Replace the stopper and allow the control to stand for 20 minutes, swirling occasionally. Before sampling, gently swirl the vials several times to ensure homogeneity. Keep the bottle undisturbed for 20 minutes.

Label 10 vials with Lot No. Date of reconstitution, expiration date and Abnormal Control, Normal Control or calibrator as needed. Label the cap with I, II or III. Care should be taken while opening the vial cap ensuring no spillage of the granules from the QC bottle. The cap should be kept safely on the side of the table and only used with its original vial.

After 20 minutes gently mix the vial and prepare aliquots. For controls, prepare 10 aliquots of 500 µl each; for calibrators, prepare 12 aliquots of 400 µl each. Before transferring liquid from the main vial, swirl the vial well to ensure a homogenous solution. Tightly seal the aliquots and place them inside the freezer. Repeat for different levels of QC or for calibrators as needed. Once the QC samples or calibrators are taken out from the deep freezer they can not be frozen and reused.

Storage and Stability

The test reagents are stable, protected from light, unopened, up to the stated expiry date when stored at 2-8°C. Opened reagents must remain refrigerated and protected from light except while being aliquoted onto the analyzer. The analyzer has onboard refrigeration so that reagents may remain on the analyzer overnight; they must be tightly recapped when not in use. Refer to Table 1 for stability for opened test reagents.

Sodium hypochlorite, HCl and Extran are good until the expiration date when stored in a cool well ventilated area at room temperature < 40°C. Once prepared for use, sodium

hypochlorite and HCl are changed every week; Extran is good for 15 days after preparation.

Controls and Calibrators are good until expiration date when stored at 2-8°C. After aliquoting, they are good for 30 days when stored at -10 °C to -20°C.

Test Reagent Calibration

Test reagents must be calibrated when a new container is opened, when reagent is aliquoted onto the analyzer, when there are QC issues or corrective action is warranted, at the discretion of the technician or when the manufacturer's open reagent stability has been exceeded. Refer to individual analyte SOP's for calibration stability.

SPECIMEN TYPES AND STABILITIES

Specimens that are unacceptable for testing must be rejected. The technician should fill out a Specimen Rejection form and send it back to the appropriate health care provider. Some specimens with issues such as mild hemolysis or lipemia may be analyzed; the patient report must include comments of the problem.

Specimens are routinely stored at room temperature after analysis until the next working day. If problems are suspected or testing cannot be completed the same day of receipt, specimens may be refrigerated or frozen for further testing. Refer to the Abnormal Results section for more information.

Specimen types and stabilities are noted in the individual analyte SOP's.

OLYMPUS AU 400 MAINTENANCE

Daily

1. Inspect the sample dispenser and reagent dispenser for leaks.
2. Inspect the detergent rolling tubes for leaks.
3. Check the amount of W1 solution.

4. Inspect and clean sample probe, reagent probe and mix bars.
5. Check the printer and paper.
6. Check the de-ionized plant. Refer to separate SOP for instructions.

Weekly (Should be completed before 11:00 AM when done)

1. Wash the sample probe, reagent probe, mix bars
2. Execute W1, W2 and photocal (Cuvette check)
    a. Alternate base and acid reagents on the instrument.
    b. From the reagent screen, press the W1 icon.
    c. Next repeat for W2.
    d. Check cuvette status.
        i. From the reagent screen press cuvette status
        ii. Check for errors
        iii. Save weekly reports
3. Wash the sample pre dilution bottle
4. Document in log sheets

Monthly, Quarterly and Annual

All other maintenance is performed by service engineers from CPC Pharmaceuticals. These visits will be documented.

Unscheduled Maintenance, QC Issues, and Troubleshooting

Any type of maintenance that is not part of scheduled service, performed either by YRG staff or by CPC service staff must also be documented. These may be as a result of QC failure, QC trends, error messages, mechanical failures, or abnormal patient results that indicate potential instrument problem. Log sheets will be kept in the chemistry department for documentation.

TESTING SAMPLES/ WORKFLOW

Morning Startup

1. Check room temperature. It should be 25°C ± 5°C.
2. Turn on Olympus AU 400 instrument. It will have a warm up procedure that takes ~20 minutes.

3. Discard samples from previous workday.
4. Clean work surfaces with appropriate anti-microbicidal substance.
5. Turn on waste and water units. Verify waste is functioning properly. Water conductivity should normalize to < 1.
6. Open Hood on analyzer.
7. Check for errors.
8. Bring daily controls (BIO RAD Lyphochek I and II) and calibrator (Olympus system Cal) aliquots to room temperature.
9. Perform and document all daily and weekly maintenance procedures as needed.
10. Once analyzer is in standby, remove reagent caps.
11. Check reagent volumes and refill when necessary. Check that no reagents have passed expiration period for open reagent containers. Document in the "Internal Indent register"
12. Discard remaining old reagent-do not mix with new.
13. Run blanks, calibrators (as needed) and controls.
    a. Main screen
        i. Calibration
            1. Click on CAL
            2. Click on specific tests to be calibrated
            3. View tube positions on screen
            4. Arrange calibrators in a yellow rack
            5. Place on the analyzer
        ii. Blank
            1. Place a tube of DI water in position 1 of a blue rack
            2. Place on the analyzer
        iii. Controls
            1. Click on QC
            2. Click on the desired tests
            3. Place level 1 in position 3 and level 2 in position 4 of a green rack
            4. Place on the analyzer

b. Click on the green arrow icon to start the analyzer
c. The system will run for 20-30 minutes
d. Go to data display to see results

14. Once these have all passed successfully, you are ready to process samples.

Sample Processing

Refer to Appendix B for Chemistry Department Workflow chart

1. Specimens will be received first in the specimen processing unit adjacent to chemistry. The specimen processing unit will deliver the appropriate specimens and test requisition forms (CCPL) to chemistry.
2. Chemistry personnel must first check that specimens are properly labeled with necessary information. They will then check that there is a corresponding CCPl with the correct information.
3. The specimen must then be evaluated for correct type and specimen integrity. Unacceptable specimens should not be processed and a specimen rejection from must be filled out and dispatched to the clinical unit. Specimens with slight problems (such as mild lipemia) may be processed and documented on the CCPL at the discretion of the technician.
4. Specific Criteria for Specimen Rejection:
    a. Wrong specimen type (Example: whole blood instead of serum)
    b. Missing or incomplete Specimen ID#
    c. Greater than mild hemolysis for tests affected by hemolysis (reject only for specific tests)
    d. Gross hemolysis (reject for all tests)
    e. Specimens that have not been properly transported and handled
    f. Specimens that are too old
    g. Insufficient volume
5. Create a test list from the CCPL's. Include the Patient ID number, accession number and tests required.
6. Recheck the test list for errors.
7. Arrange the samples in a white rack to correspond to the test list.

8. Program the AU 400.
    a. Go to the User drop down menu
    b. Click on Normal
    c. Enter the ID's for the patients to be tested
    d. Place the tubes in a grey rack in the same order.
    e. Recheck the program against the order of the tubes in the rack.
9. Place the rack in the AU 400 by pressing the green icon.
10. Start the AU 400.
11. The AU 400 will generate values after analysis. They may be printed if needed for source documents for specific studies or as desired.
12. All the results will be in the data display screen
13. To view individual patient results
    a. Go to User
    b. Click on Data Edit
    c. Click on routine
    d. Locate the Specimen ID
14. Check all results. Highly abnormal results or results outside the linearity of the AU 400 must be evaluated. See the "Abnormal Results" section.
15. To repeat tests on a sample
    a. From the main menu click on routine
    b. Click on test requisition
    c. Click on repeat mode
    d. Find the previously tested sample
    e. Click on the tests desired to be repeated
    f. Place the sample in the corresponding position in an orange rack
16. All transcription of results must be verified. This includes transcription from the screen to the log book and from the log book to the CCPL forms. The log book is the source document for all results from the AU 400 unless defined differently for specific research studies.

17. Once completed CCPL's have been checked and verified, the forms are sent to the data processing unit to enter the results into the Hospital Management System and generate computerized result forms.
18. The computerized result forms are sent back to chemistry to be rechecked against the original CCPL forms. Once verified, the computerized result forms are sent to the clinical units.
19. Refer to the current YRG CARE Infectious Disease Laboratory Critical Values SOP for guidelines for handling critical values. All technicians must be familiar with critical values and a list should be accessible in the laboratory.

20. During the course of the day, monitor reagent levels.
    a. From the main menu, click on the Olympus AU 400 Icon
    b. Click on checked
    c. Click on position display
    d. The analyzer will display remaining reagents and shots for each analyte.
21. The instrument is shut down at the end of each work day. The refrigeration unit for the reagents should remain on. Monday to Friday, this will take place around 6:00 PM. On Saturdays, the technician should wait until 6:30 PM. This time is flexible based on specimens.

REPORTABLE RANGE (LINEARITY)

Each analyte has a defined reportable range or linear range. When a specimen's initial result is higher than the reportable range, retest the specimen. If the results agree, the specimen should be diluted and retested. The diluted result must then be multiplied by the dilution factor to get the actual result. The result obtained from the analyzer (before multiplication by dilution factor) must be within the reportable range or a higher dilution factor may be warranted.

Specific information regarding types of diluents, dilution procedures and dilution limits should be obtained from the appropriate package inserts.

In certain cases it will be impossible to generate a result through dilutions. In these cases, the result should be given as "greater than X" with X referring to the upper limit of the reportable range.

When the initial test result is less than the lower limit of the reportable range, the specimen should be retested. If the results agree the, result should be given as "less than Y" with Y referring to the lower limit of the reportable range.

In all cases, technician judgment must be used when results fall outside the reportable range. In cases where it is possible the specimen has been contaminated or other issues with specimen integrity are apparent, the specimen should be rejected.

Refer to analyte SOP's for specific linearities.

ABNORMAL RESULTS

1. Recheck specimen integrity (clotted specimen, hemolysis etc….)
2. If analyzer performance is in doubt, perform corrective action or rerun a control. Analyzer performance should be evaluated if many specimens yield abnormal or highly abnormal results when not expected.
3. If steps 1 and 2 are acceptable, repeat the analysis
4. If result is outside linear range, perform dilution based analysis when appropriate. See Linearity section and package inserts for more information.
5. Consult a supervisor or second technician when needed.
6. If clinical information is available, this can be used to evaluate if the result is biologically plausible.
7. When the validity of the result does not make clinical sense or is in doubt, do not report results and request a new sample. Communicate with clinician the issues.
8. Provide proper documentation as to actions taken.
9. Abnormal specimens should be retained for 30 days frozen at <-12° in case of requests for further testing.

REFERENCE RANGES

Reference ranges (also known as normal ranges or intervals) are used by clinicians to evaluate results from quantitative diagnostic laboratory tests. Laboratory technicians should also be aware of these ranges so that the can evaluate if results obtained make biological sense. Refer to analyte SOP's for a list of reference ranges for specific tests.

TURN AROUND TIME

- Turn around time (TAT) for chemistry is defined as when the sample is received from the specimen processing department to when the result is given to the data clerks.
- Routine TAT is set at ≤ 3 hours.
- When specimens are received with comments indicating they are needed urgently or stat, the TAT is set at ≤ 1 hour.
- Refer to the current version of the YRG CARE SOP for critical values for special handling in cases of certain values extremely outside of normal ranges.
- Conditions which delay reporting of results should be documented and communicated to laboratory mangers and clinics as needed.

CRITICAL VALUES

Table 1 lists critical values in chemistry. Immediate action must be taken in these cases. All chemistry technicians must be aware of these values and take appropriate action as described in the version of the Chemistry Critical Values SOP.

Table 1. Chemistry Critical Values

Parameter	Lower limit	Upper limit
Glucose	< 40 mg/dL	> 450 mg/dL
Potassium	< 2.8 mmol/L	> 6.2 mmol/L
Sodium	< 120 mmol/L	> 160 mmol/L
Calcium	< 6.0 mg/dL	> 13 mg / dL
Creatine Kinase	N/A	> 1000 U/L
Magnesium	< 0.9 mg/dL	> 3.7 mg/dL
Urea	N/A	> 85 mg/dL

Albumin		
SOP Number:	Total Number of Pages: 12	
Primary Author: L. Ameedha / P.Nandagopal	Signature & Date:	
Reviewed by: M. Sundaram, E. Livant	Signature & Date:	
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PURPOSE

This SOP is intended to give detailed information about the Albumin assay performed at the YRG CARE Infectious Disease Laboratory. This is to ensure that technicians performing the test have all the necessary information to accurately measure this analyte and to report results.

SCOPE

This SOP applies to Albumin testing performed in YRG CARE Infectious Disease Laboratory using the Olympus AU400. This SOP does not replace complete training and only authorized personnel should perform testing using the AU 400. The AU 400 SOP must be read before reading this SOP.

All human specimens should be treated as infectious. Refer to the current YRG CARE Safety SOP for important safety information before handling specimens.

PRINCIPLE

A colored complex is formed when bromocresol green reacts with albumin. Absorbance of the albumin-BCG complex is measured bichromatically (600/800nm) and is proportional to the albumin concentration in the sample. The results are reported in units of g/dL.

CLINICAL SIGNIFICANCE

Albumin is the most abundant protein in human plasma, representing 55-65% of the total protein. Its primary biological functions are to transport and store a wide variety of ligands, to maintain the plasma oncotic pressure and to serve as a source of endogenous amino acids. Albumin binds and solubilises non-polar compounds such as plasma bilirubin and long chain fatty acids as well as binding numerous pharmaceuticals.
Hyperalbuminemia is infrequent and is caused by severe dehydration and excessive venous stasis.

Hypoalbuminemia may be caused by impaired synthesis e.g., in liver disease or in protein deficient diets, increased catabolism as a result of tissue damage and inflammation; reduced absorption of amino acids caused by malabsorption or malnutrition; protein loss to the exterior as observed in nephrotic syndrome, enteropathy or burns; and altered distribution e.g. in ascites. Severe hypoalbuminemia results in a serious imbalance of intravascular oncotic pressure causing the development of edema.

Measurements of albumin concentrations are vital to the understanding and interpretation of calcium and magnesium levels because these ions are bound to albumin, and so decreases of albumin are also directly responsible for depression of their concentrations.

QUALITY CONTROL

Refer to the Olympus AU 400 SOP

REAGENT

Catalog # OSR 6102. No special preparation needed. Must be recalibrated (Olympus system Calibrator CAT #66300) at least every 30 days (see Olympus SOP for additional information). May be used for 30 days after opening.

EQUIPMENT

Refer to the Olympus AU 400 SOP

SPECIMEN

Serum from plain (no additive) or serum separator tubes may be used. It should be separated from the cells as soon as possible. It may be stored for 2-8°C for 30 days or 15-25°C for 7 days.

LINEARITY

1.5 – 6 g/dL

NORMAL RANGE

4.2-5.2 g/dL

CRITICAL VALUES

N/A

REFERENCES

Olympus AU 400 User's manual

Reagent Package Insert

YRG CARE Biochemistry Validation Reports

YRG CARE Normal Range Study Report

Establishing New Control Lot Means and Quality Control (QC) Ranges Through Parallel Testing and Historic Coefficient of Variation (%CV_h)

In order to optimize controls, it is important for clinical laboratories to establish their own means and QC ranges for each new control lot number. Using the manufacturer's QC ranges will waste you QC money, as the ranges will be too wide to provide an appropriate level of warning value. Assay ranges for new control lot numbers should be confirmed prior to the expiration of the old lot. The means for all analytes of the new lot should fall within the assay ranges provided by the manufacturer. If they do not, then something in the system needs corrective action.

If your laboratory has been using the manufacturer's ranges, but you want to improve your QC effectiveness, then following the recommendations below will help. It is strongly advised that you gradually implement changes. Pick one or two analytes to change until you are comfortable with the results, and then add one or two more analytes. Also, narrow the ranges gradually. The goal should be QC ranges based on the Standard Deviation (SD) calculated from your historic CV_h, but it might be better to narrow a little at a time, until you reach this goal. This gradual approach will reduce complications.

Three other side effects of this approach are worth mentioning. 1) Levey-Jennings charts will show much more variation with narrow SDs. This is normal and will enable the laboratory to spot problems earlier. A flat looking LJ chart is often an indication that SDs are too wide. 2) The laboratory will likely consume more calibrator, reagent, QC materials and other consumables, as more problems will be detected and corrected. Inventories of these items should be monitored carefully. 3) The increased level of error detection will give more out of control situations which will lead to more documentation of corrective action taken.

PART I: Recommendations for establishing means through parallel testing of new chemistry control lots

1. **Parallel testing** is running the current and new QC material together. Run the current QC lot and verify that the QC material is within range. Then run the new QC lot 2 to 3 times throughout the day ideally for a minimum of seven to ten days before the old lot expires. Collect at least 20 data points.

NOTE: If you observe any problems as you parallel test your controls, such as a trend, stop running the new lot number and investigate to solve the problem. A trend is here defined as six data points in one direction, i.e. steady increase or steady decrease. If the old lot expires and you need to start the new lot, then use the manufacturer's ranges until you can establish your own.

2. Review the data to ensure that there are no trends and that the precision or CV is acceptable according to the manufacturer's recommendations (available in the

instrument or method documentation). If the CV is unacceptable then you must investigate the cause, correct it and recollect the data.

3. Calculate the new means and experimental SDs for each chemistry analyte. Utilize instrument software programs or EXCEL spreadsheets to perform the calculations, if available. Or sum the values and divide by the number of values.

4. Reject any obvious outliers (data points more than 3 times the SD from the mean). After any data rejection, recalculate. Do not include rejected data points in any statistical calculations.

PART II: Establishing historical CV (CV_h)

CV_h is the historical instrument precision for that method measured as a percent (%CV = SD/Mean X 100). It can be used to establish Standard Deviation (SD), QC ranges and to monitor your instrument precision.

The CV_h is best established over several lots (3 or more) and can either be the average CV or more preferable, the highest acceptable CV over that period. CVs obtained during times of instrument malfunction or significantly higher than the average CV should not be used in calculations. If you have no previous CV data, then use the current CV as the CV_h basis and modify this value as you move on to subsequent new lots. This basis value will need to be increased slightly (~1-2%) in order to establish QC ranges that are not too narrow.

PART III: Recommendations for establishing QC ranges using CV_h

1. From the data created by the parallel testing in Part I above, calculate the CV and SD for the new control. Standard Deviation is "s" in the following example formula. It is much easier to use a calculator or an EXCEL spreadsheet for this calculation.

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

2. The calculated new lot CV must be comparable to the manufacturer's instrument-method CV and should approximate the CV_h. If it is not, then there is a precision problem with your instrument.

3. If you use the SD calculated directly from the new control data to establish your new QC ranges, you may find that these ranges are too narrow. Because they were collected over a short time period and with limited data, they do not represent enough variation in a system. It is better practice to use the CV_h, as this will accommodate lot-to-lot variation.

4. Calculate the new control range by using the new mean with the SD determined by the equation (SD = CV_h /100 X Mean). The number of SDs on either side of the mean can vary, but in many laboratories the QC range will be +/- 2 SD of the mean. See the examples of how to calculate these parameters below.

QC ranges established by this method will be more sensitive to changes and will thus alert laboratory personnel, when there are changes in a testing system . It is our goal to detect these changes before they become clinically significant and affect patient results.

NOTE: Tracking CV from lot-to-lot and month-to-month will alert staff to instrument problems. The precision is a very good indicator of the instrument's overall function. Increased CV must be corrected as soon as possible through instrument maintenance or servicing. The manufacturer's representative will not be inclined to service an instrument until the CV exceeds the manufacturer's claims, but your laboratory maybe able to correct problems before then.

PART IV: Comparing your CV_h with Manufacturer's CVs and Peer CVs

It is important to compare your CV_h to other sources. Too large a CV will not allow the QC material to be the warning signal you want it to be. Using smaller CVs will improve survey performance. Too small a CV will cause more QC errors wasting material and time unnecessarily.

1. The first comparison of your CV_h should be made to the specifications of the instrument manufacturer. This information is generally contained in the instrument manual or the method documentation. If your CV_h is higher than those specified, then service needs to be called. CVs are generally higher when the mean of the analyte is at a low value. This can lead to misinterpretation so some analyzer manufacturers publish acceptable SDs at the lower levels rather than CV.

2. Some labs like to compare their CV_h with the average CV from CAP or other External Quality Control (EQA) Provider peer for each analyte. Generally, the laboratory's CV should be less than the EQA CV, since the peer group will encompass many more variables. EQA CVs are calculated for each sample by dividing the survey peer sample SD by the sample mean and multiplying by 100 to arrive at the sample %CV. This is done for each analyte sample on the survey. Then all samples are either averaged or the highest sample CV for the analyte is compared with your laboratory's CV. Ideally, your lab should be less that the EQA CV. If it is not, then you should investigate why your system is unable to recover the precision of your peers.

3. Some Quality Control material companies offer a peer group program. This is a program in which your lab submits their mean, SD and CV data each month. The company then compiles the data with other labs using the same QC material, instrument and method and publishes this data. This peer data is very helpful in allowing you to judge your instrument's performance on a monthly basis. You

would prefer to have your CV less than the peer's CV. Contact your QC material supplier to identify what might be available.

PART V: Example

New normal and abnormal controls have arrived a month before the old lot expires or runs out. The laboratory was able to obtain enough new control to last until its expiration date. The lab staff ran the new lot of controls in parallel with the old lot each day until they had 20 data points. They then calculated the new mean for each control and for each analyte.

Glucose Normal Control Mean
The manufacturer in their control package insert stated that the glucose normal control mean should be between 80 -92. The lab recovered a mean of 85 from the parallel testing above. The mean is within the manufacturer QC range and this became the new normal control's glucose mean.

Glucose Normal Control SD
The manufacturer of Analyzer XYZ states that their instrument's precision for this glucose method is 4.0%. The last three lots of the normal control had CVs of 2.2, 1.8 and 2.5. These numbers show that the instrument-method precision is performing as designed.

Even though we will not be using the CV from the parallel studies, it is important to check that it is comparable to (less than) that of the historic CV.

The historic average CV is 2.2 and the historic highest CV is 2.5. The EQA peer group in this control range had a CV of 3.1. The laboratory also participates in a peer group program with the control vendor. The peer group for the new control and instrument-method combination runs a CV of approximately 2.7. It was decided to use 2.5 as the historic CV_h.

The lab used this formula to calculate their new SD goal

$$\mathbf{SD = \frac{CV_h \text{ X mean}}{100}}$$

Glucose Normal **Lot 001** Mean = 85 mg/dL, $CV_h = 2.5$

$$\mathbf{SD = \frac{2.5 \text{ X } 85}{100} = 2.1}$$

The laboratory uses a +/- 2 SD range for this assay, so the control range became: **Range = Mean +/- 2 SD (85.0 +/- 4.2 or 80.8 – 89.2)**. This range was monitored and adjusted, if needed, as more data was accumulated. A record of all changes to the QC settings in the

instrument is kept in a log, and any aliquoting of QC is also logged to monitor stability of the control.

The laboratory would then go through the same steps for the abnormal control. The CVs will be different for each control and each analyte.

Appendix I –Roche Description of how to run parallel testing or precontrols on the the Cobas Integra. *Instructions obtained from Roche website.*

How do I define and measure precontrols?

The QC parameters for precision controls are assigned mean and standard deviation by the laboratory. These values have to be established beforehand in a precontrol period before the precision control can be used in the daily routine. Over a period of time, the precontrol is measured in regular intervals. At the end of the precontrol period, the measured results are used to calculate the assigned mean and standard deviation for all tests that have been included in the precontrol measurements.
The process of measuring precontrols and calculating QC values is as follows:

1. At the beginning of the precontrol period, the control ID and the control lot are entered, and a precontrol request is created in the Create Precontrol Request window.
2. During the precontrol period, regular precontrol measurements are requested in the Append Pre-Control Request window. These requests include all tests for which QC values have to be established.
3. At the end of the precontrol period, the QC values are calculated for all measured tests and read-into the Control Lot window.

To create a precontrol request for a new control lot

1. Open the Quality Control work area.
2. Choose Request > Pre-Control > Create. The Create Pre-Control Request window is displayed.
3. Enter the control ID (short name), the lot number of the precontrol, and its rack and cup position.
4. Select the tests for which you want to establish the QC values.
5. Click OK. A precontrol request is scheduled, including all selected tests.
6. Place the precontrol in the rack and cup position defined in the Create Pre-Control Request window.

All precontrol requests have the prefix "$P_" in the order ID (for example $P_RON) and are listed in the Orders work area in the View By Orders and View By Patient lists. Precontrols are not listed in the View Cal/QC list of the Orders work area.

To continue precontrol requests during the precontrol period

1. Open the Quality Control work area.
2. Choose View > By Pre-Controls and select the precontrol you want to order.

3. Choose Request > Pre-Control > Continue. The Append Pre-Control Request window is displayed.
4. Enter the rack and cup position of the precontrol.
5. Click OK. A new precontrol request is scheduled.
6. Place the precontrol in the rack and cup position defined in the Append Pre-Control Request window.

To read-in QC values from precontrols at the end of the precontrol period

1. Open the Configuration work area.
2. Choose Definitions > Controls.
3. Select the control from which you want to read-in precontrol results.
4. Choose Modify > Pre-QC. The Control Lot window is displayed.
5. Click Read Pre QC to read-in all QC values of all tests listed in the Test List of the Control Lot window. The assigned mean and assigned standard deviation values are automatically calculated and read-in for all tests included in the precontrol measurements.

To read-in only selected tests from the precontrol measurement, choose Modify > Lot, select the test in the Test List and click Read Pre QC.

Section 12: Coagulation

1. Overview: The process of blood clotting and the subsequent dissolution of the clot following repair of the injured tissue, is termed hemostasis. Coagulation tests monitor hemostasis. Many coagulation tests measure the time it takes for an individual's blood to clot under standardized conditions; some may measure directly clotting factors in the blood. Frequently, this is done to screen and monitor genetic conditions affecting hemostasis and to monitor anticoagulant therapy. It may also be done to monitor other conditions, such as liver disease which may affect a patient's ability to form clots. Several of the microbicide gels have an anticoagulant affect and for patient safety reasons may require the monitoring of coagulation tests.

   For HPTN studies, the main tests performed are the Prothrombin Time (PT), the International Normalized Ratio (INR), and the Activated Partial Thromboplastin Time (aPTT, APTT or PTT). The PT and INR monitor the extrinsic coagulation pathway and the APTT monitors the intrinsic coagulation pathway.

2. Specimen: The primary specimen for coagulation testing is sodium citrate (NCCLS recommends using 3.2 % Sodium Citrate) plasma. Follow manufacturer's guidelines for specimen criteria regarding storage conditions; usually the PT must be performed within 2 to 4 hours of collection unless the specimen is frozen. Coagulation results will be erroneous when these guidelines are not followed.

3. Internal Quality Control:
   a. Establishing local QC ranges: each site must calculate its own mean and standard deviations based on parallel testing. At least 20 runs are required. The new lot should be run in parallel with the old lot to ensure instrument performance while establishing the new ranges.
   b. Levels required: laboratories must run at least a normal and a high abnormal level.
   c. Frequency: 2 levels must be run and satisfactory by multi rule QC each day the analyzer is used. Another control should be run each additional 8 hours of testing on that day.

4. External Quality Control: Each HPTN/MTN site must run the appropriate CAP coagulation basic panel. Additional surveys may be added as desired.

5. Calibration: Most coagulation analyzers do not require calibration by the laboratory.

6. Specimen Analysis: Particular attention must be paid to the INR. There are regional differences in the strengths of reagents (thromboplastin) used for the PT which make it difficult to compare results from different areas. Recognizing this, the World Health Organization (WHO) created the International Sensitivity Index (ISI), which is a correction factor based on the response of different thromboplastins. The formula for the INR:

$$INR = (\text{Patient PT}/\text{Normal PT mean})\ ISI$$

Every lot of PT thromboplastin will contain an ISI. Each lab will establish its own normal PT mean based on the local population. It is extremely important to check the ISI with each new lot of thromboplastin to verify if the ISI has changed. If it has, it must be updated in the formula used by the lab to calculate the INR. Each new ISI value requires verification of the normal range as well as a calculation of the geometric mean. Each lab needs to enter the geometric mean as well as revised ISI value before running patient samples with the new reagent. Failure to do this will lead to erroneous INR results.

7. Microscopy: N/A

8. Troubleshooting Specimens:
    a. Clotted samples-never use clotted samples. A new sample must be obtained.
    b. Hemolysed samples-in cases of more than slight hemolysis, redraw the specimen.

9. Maintenance: Most coagulation analyzers require only minor daily and weekly maintenance such as cleaning. It may be necessary to change lamps on an annual basis. Follow manufacturer's recommendations.

Kilimanjaro Reproductive Health Programme
Laboratory Standard Operating Procedure

SOP Number: (TBD)	**Title:** Coagulation Tests
Original Effective Date: DRAFT	**Revision Effective Date:** Not Applicable

Purpose:

Coagulation tests measure the time it takes for an individual's blood to clot under standardized conditions. Frequently, this is done to screen for and monitor genetic conditions affecting clotting and to monitor anticoagulant therapy. It may also be done to monitor other conditions, such as liver disease which may affect a patient's ability to form clots.

Scope:

This procedure applies to all coagulation testing for KRHP laboratories. The current test menu includes The Prothrombin Time (PT), The International Normalized Ratio (INR), and the Activated Partial Thromboplastin Time (aPTT, APTT or PTT).

Principle:

The Vital Scientific Fibron-1

The Fibron-I is a single channel coagulometer that works on the principle pf photometric clot detection. The detection of the coagulation end point is achieved by measuring the change in optical density of a plasma sample. Light from a high intensity lamp is passed through the plasma and reagent sample and the transmittance is determined by a photosensitive detector.

Clinical Significance:

Coagulation assays are used to evaluate the patient's potential for hemostasis (the stoppage of bleeding) or to monitor anticoagulant therapy. Hemostasis has three main components: vasoconstriction, the clotting cascade and platelets. These tests monitor the clotting cascade. The clotting cascade is a series of factors in the blood which activate after injury with the final result being the formation of a fibrin mesh which catches platelets in an attempt to seal the opening and prevent blood loss. There are two main pathways for the coagulation cascade: intrinsic and extrinsic.

Genetic conditions such as Von Willebrand disease, hemophilia, factor deficiencies or Vitamin K deficiency can interfere with hemostasis. In theses conditions, the PT and/or APTT may be prolonged. Acute liver damage or DIC will prolong both the PT and APTT and cause a decrease in platelets. The PT and APTT are generally screening tests and further testing such as thrombin times and factor assays may be needed for accurate diagnosis. The PT monitors the extrinsic pathway and the APTT monitors the intrinsic pathway.

Anticoagulant therapy is commonly used when it is necessary to reduce the patient's tendency towards clotting. Examples include situations involving myocardial infarction, stroke, embolisms, atherosclerosis and gangrene. Anticoagulant therapy can be short or long term by numerous routes of administration. Some oral anti-coagulants such as coumadin will generally increase the PT. Intravenous anticoagulants such as heparin will have more of an affect on the APTT but the PT is frequently elevated as well.

The INR is a standardized measure used to facilitate comparability between laboratories. It takes into account the normal patient range at a certain laboratory, the strength of the lot or reagent used and the PT result. It is generally used for monitoring oral anticoagulant therapy.

Decreased PT, INR and APTT results are not generally clinically significant.

Specimen:

Sodium Citrate Plasma, fresh within 4 hours of collection is the preferred specimen. Fresh specimens should be refrigerated while awaiting testing. If testing is delayed beyond 4 hours, plasma must be separated from cells and frozen at -15º or lower. Frozen samples may be stored for 2 weeks and should be fully thawed and mixed before testing.

Reagents:

REAGENT STORAGE AND PREPARATION				
Assay	**Working Reagent Components**	**Storage of Unopened Reagents**	**Working Reagent Preparation**	**Stability and Storage of Working Reagent**
PT	1] Thromboplastin 2] Buffer	2ºC-8ºC	Add one bottle of buffer to one bottle thromboplastin	5 Days at 2ºC-8ºC
APTT	1] Cephalin	2ºC-8ºC	Add 9.0 ml DI H_2O to one bottle Cephalin	7 Days at 2ºC-8ºC
APTT	2] Calcium Chloride	2ºC-8ºC	Ready to use	Expiration date on bottle
Controls (2 Levels)	1] Reagent Vials	2ºC-8ºC	Add 1.0 ml DI H_2O to one reagent bottle	24 hours at 2ºC-8ºC

Equipment and materials:

- Equipment
  - Vital Lab Fibron-I analyzer
  - 200 µl adjustable pipette. It is preferable to have 2.
- Materials
  - Pipette tips
  - Cuvettes

Quality control:

2 levels of control, normal and high abnormal, should be run each day testing is performed. If testing exceeds 8 hours, 1 level of control should be run each subsequent 8 hours.

Control results must meet KRHP specific Multirule criteria before reporting of patient results. Appropriate training must be given to understand these criteria. The criteria are:

- ✓ Control outside 2 Standard Deviations (SD) of the mean (1_{2S})
- ✓ Control outside 3 SD (1_{3S})
- ✓ Two consecutive runs outside 2SD (2_{2S})
- ✓ Four runs to greater then 1SD from the mean on one side (R_{4S})
- ✓ Run is 4 SD from previous run (4_{SD})
- ✓ Seven runs in one direction, up or down (7_{γ})
- ✓ 10 Consecutive runs to one side of the mean

See Appendix A for flowchart.

Always ensure that control limits used for evaluation are for the correct lot of control. Refer to package inserts.

Procedure Stepwise:

1. Turn on analyzer. The analyzer will say "Warming up". This will take 15 minutes.
2. The analyzer will say "Auto Start?" Toggle between yes and no to select option desired. Press enter.
3. The analyzer will say "Select Test:" Toggle between PT and APTT. Do not select open test. Press enter.
4. Procedure: <u>PT Auto Start On</u>
   - 4.1. If you with to have the instrument automatically calculate the INR, toggle the star to INR and press enter. Ensure that the ISI and patient normal range are correct.
   - 4.2. If you do not wish the instrument to calculate the INR, toggle the star to PT.
   - 4.3. Place a sufficient volume of PT reagent in the heating block for 10-30 minutes.
   - 4.4. Place 100 µl of patient or control into a cuvette and place the cuvette in the heating block for 2-20 minutes.
   - 4.5. The analyzer will read "Transfer Cuvette and press enter". Put the patient or control cuvette into the reading well and press enter.
   - 4.6. Pipette 200 µl of PT reagent into the cuvette.
   - 4.7. The timer will start automatically. When a clot is detected, the timer will stop and say "Clotted".
   - 4.8. If just reporting PT, result will print automatically. If also running, press enter. A result will print.
   - 4.9. Press enter again. The analyzer will ask "PT: Test again?" If you wish to continue testing toggle the star to Yes and enter. If PT testing is finished, toggle to No and enter.
5. Procedure: <u>PT Auto Start Off</u>

5.1. If you with to have the instrument automatically calculate the INR, toggle the star to INR and press enter. Ensure that the ISI and patient normal range are correct.

5.2. If you do not wish the instrument to calculate the INR, toggle the star to PT.

5.3. Place a sufficient volume of PT reagent in the heating block for 10-30 minutes.

5.4. Place 100 µl of patient or control into a cuvette and place the cuvette in the heating block for 2-20 minutes.

5.5. The analyzer will read "Add PT reagent and press enter". Put the patient or control cuvette into the reading well and press enter.

5.6. Pipette 200 µl of PT reagent into the cuvette. Press enter.

5.7. The timer will start. When a clot is detected, the timer will stop and say "Clotted".

5.8. 4.8. If just reporting PT, result will print automatically. If also running, press enter. A result will print.

5.9. Press enter again. The analyzer will ask "PT: Test again?" If you wish to continue testing toggle the star to Yes and enter. If PT testing is finished, toggle to No and enter.

6. Procedure: APTT Auto Start On:

6.1. The analyzer will display "Incubation Time: 180 seconds" Press enter.

6.2. Place sufficient Calcium Chloride reagent in the heating block for 10-60 minutes.

6.3. The analyzer will read "ADD APTT REAGENT Press Enter". Place a cuvette in the measuring well. Add 100 µl patient or control and then add 100 µl APTT reagent. Press enter.

6.4. The counter will go for 180 seconds. It will start beeping at 9 seconds.

6.5. When the timer reaches 0, add 100 µl calcium chloride. The timer will start automatically.

6.6. When a clot is detected, a result will print. Press enter.

6.7. The analyzer will read "APTT: Test again?" Toggle to yes or no if further testing is desired.

7. Procedure: APTT Auto Start Off:

7.1. The analyzer will display "Incubation Time: 180 seconds" Press enter.

7.2. Place sufficient Calcium Chloride reagent in the heating block for 10-60 minutes.

7.3. The analyzer will read "ADD APTT REAGENT Press Enter". Place a cuvette in the measuring well. Add 100 µl patient or control and then add 100 µl APTT reagent. Press enter.

7.4. The counter will go for 180 seconds. It will start beeping at 9 seconds.

7.5. When the timer reaches 0, add 100 µl calcium chloride. Press enter.

7.6. When a clot is detected, a result will print.

7.7. The analyzer will read "APTT: Test again?" Toggle to yes or no if further testing is desired.

Reporting of Results:

Report Printouts should be fixed onto KRHP study specific Coagulation Testing Source Document Forms. Then results should be written in on these forms. PT and APTT results will be reported in seconds. INR results are a percentage.

Calculations:

The Fibron will automatically calculate the INR if desired. Three components are included in the INR:

- ✓ The PT result
- ✓ The International Standardized Index (ISI). This is a factor that relates to the strength of the lot of reagent. This information must be checked with each lot of reagent and updated if necessary.
- ✓ The normal PT geometric mean for the local population. This must be determined by a reference range study of at least 20 persons for each new lot of reagent.

The formula is

$$INR = (\text{Patient PT}/\text{Normal PT mean})^{ISI}$$

Abnormal Result Handling

PT results greater than 120 seconds and APTT results greater than 150 seconds will not print a result. The analyzer will display "No clot detected."

- ✓ Rerun sample.
- ✓ Check the cuvette visually for a clot.
- ✓ If more than one sample is not clotting, rerun controls.
- ✓ There may be a problem with the sample. Request a new sample.

Expected Results-Normal Ranges: TBD

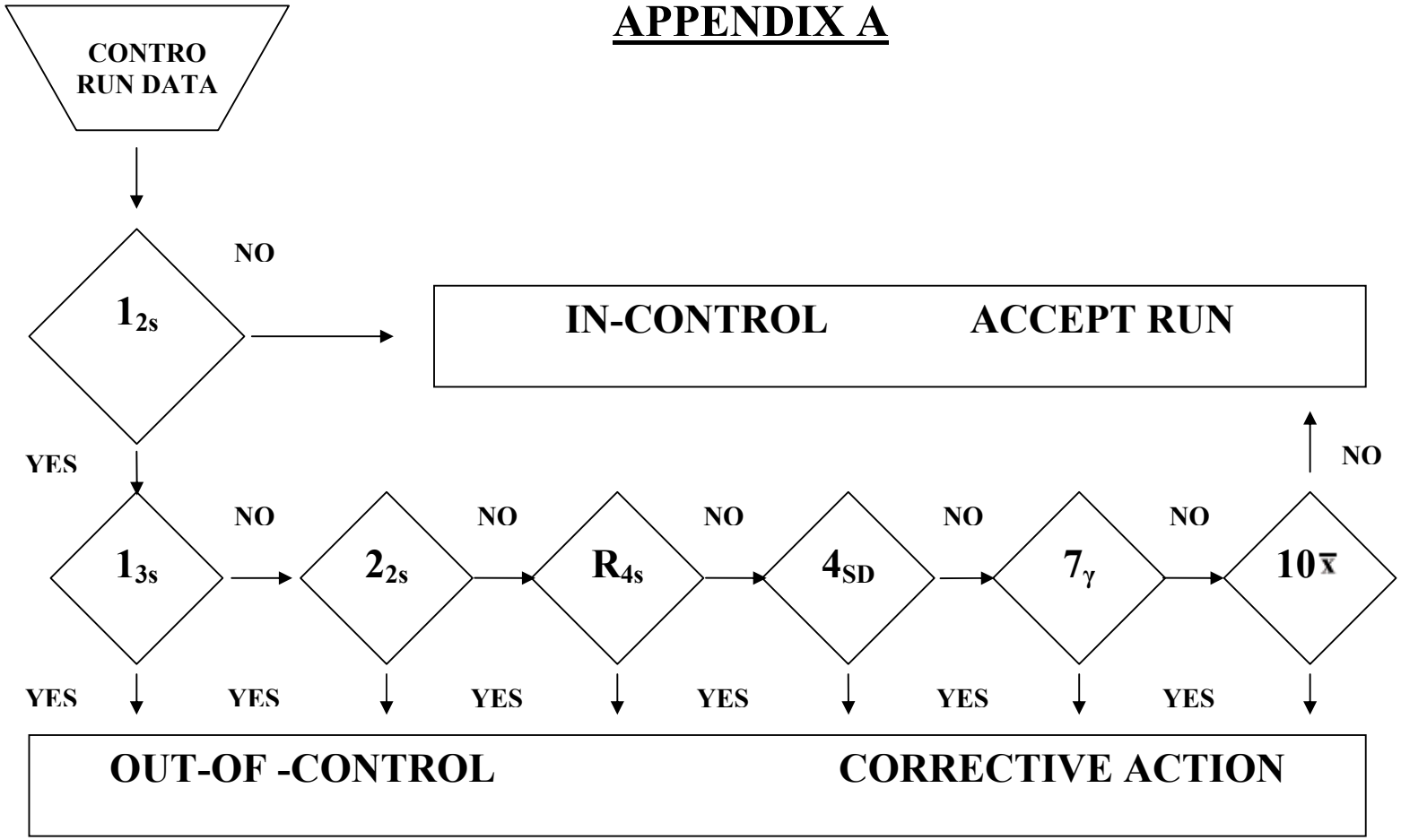
Instrument Maintenance:

There is no scheduled maintenance. Keep the machine clean especially the measuring well. Keep the analyzer covered when not in use.

References:

Vital Scientific Fibron I Blood Coagulation Analyzer Operating Manual. Hemosil reagent package inserts.

APPENDIX A


CONTRO
RUN DATA
NO
1_{2s}
IN-CONTROL ACCEPT RUN
YES
NO
1_{3s}
NO
2_{2s}
NO
R_{4s}
NO
4_{SD}
NO
7_{γ}
NO
$10_{\bar{x}}$
YES
YES
YES
YES
YES
YES
OUT-OF -CONTROL CORRECTIVE ACTION

Section 13: Flow Cytometry

1. Overview: monitoring of CD4 and CD8 lymphocytes is crucial in the diagnosis and treatment of HIV infected patients. Many HPTN/MTN studies require CD4 (and sometimes CD8) measurements. The absolute CD4 count is most relevant for adult populations. For pediatrics, the CD4 percentage is frequently used.

   The most common way of measuring CD4 and CD8 lymphocytes is through Flow Cytometry. The following is a very brief overview of flow cytometry with some sample SOP's. Many non-US sites use (but are not limited to) the BD FACSCount; thus most of these SOP's are for the BD FACSCount and may be adapted for individual sites. Also included is a list of questions and answers from the IQA group, which provides technical support for flow cytometry.

2. Specimen: most CD4/CD8 analysis is done using EDTA whole blood (K_2 EDTA is recommended). Specimen stability is generally ~48 hours but some new techniques (such as PLG) may have longer stabilities; follow manufacturer's instructions as well as DAIDS requirements.

3. Internal Quality Control: QC must be done each day testing is performed following at least minimum guidelines from the manufacturer. Additional QC may be implemented as desired by the site. If more than one instrument is used, a sample should be run daily on each instrument to check for instrument correlation. The tolerance limits of the monitoring parameters should be previously defined by instrument correlation studies.

4. External Quality Control: HPTN/MTN sites will participate in the United Kingdom National External Quality Assurance Service (UKNEQAS) proficiency testing. They may choose to also participate in other additional programs.

5. Calibration: many flow cytometers will require calibration beads which must be run each day of use with a normal human sample.

6. Specimen Analysis: Dual vs. Single Platform- When a single tube of blood is analyzed on a single instrument to generate absolute lymphocyte subsets, this is referred to as "single platform". If two instruments are used (a flow cytometer and hematology analyzer) to derive the absolute count, this is referred to as "dual platform". This terminology does not apply to derivation of percent values.

7. Microscopy: Some manual methods exist which involve staining of cells and counting using a microscope. These are not in use in any HPTN/MTN studies.

8. Troubleshooting Specimens: clotted specimens cannot be used and must be redrawn.

9. Maintenance: most flow cytometers have lasers which are precisely calibrated; moving the instrument may require realignment of the laser. More simple systems such as the BD FACSCount will have minimal maintenance procedures. More complex systems such as the BD FACSCalibur will require more attention. Follow manufacturer's recommendations/.

Questions and Answers for CD4 flow Cytometry

IQA guidelines

On the FACSCount report, do the sites need to keep track and plot any of the following Slope intercept R values?

The acceptability of these statistical values is embedded in the FACSCount software and the software will trigger a failure if these values are outside of the defined acceptable limits. Since the software is monitoring these parameters for acceptability, it is not necessary to manually plot these values for tracking purposes.

What is the recommended allowable difference in absolute count between 2 FACSCounts, 2 similar Flow cytometers, and /or between FACSCount and another flow cytometer?

We recommend that each lab define the acceptable limits of variability for each analyte within the guidelines outlined by the manufacturer. Our testing shows variations within 10% for the CD3, CD4, and CD8 parameters when testing normal samples between different FACSCount instruments, between flow cytometers, and between FACSCount and other instrument/
methodology. Out testing shows variations within 15% when testing samples with CD4 counts below 200.

What do you do if you do not have a normal donor each day that you need to run your instruments?

If a normal donor is not available each day the instrument is run, a previously tested patient sample that displays marker values within normal ranges may be substituted. This specimen must be used within the pre-defined limits of sample stability for the testing system. This alternative testing method may also be applied to satisfy the requirement for a daily CD4 low control. A patient sample with low CD4 level (<200) may be selected as the CD4 low control.

If you need to refer a sample to a referral lab that uses the same analyzer, would you recommend that the primary sample be sent or would it be better to prepare and fix the sample?

Samples should be sent as an unprocessed specimen to control for variables between testing sites unless a study was conducted between the sites to show correlation of testing prepared and fixed samples sent from a primary lab. It is important that should the need arise to send a sample to a referral lab that the sample is received at the referral site within the defined stability limits of the specimen.

Can you provide a list of all websites/companies to order the CD4 controls from?

Note that some control materials may not be compatible all testing systems.

Beckman Coulter Cytometry www.beckman.com Immunotrol Cells, CD4 Normal
Immunotrol Low Cells, CD4 Low

Becton Dickinson ImmunoCytometry Systems www.bdbiosciences.com
Multi-Check Control, CD4 Normal Control
Multi-Check Control, CD4 Low Control
BD Multi-Check controls may not be compatible with the Beckman Coulter Q-Prep Cytometry System.

Streck Labs www.streck.com
CD Chex Plus, CD4 Normal
CD Chex Plus, CD4 Low
CD Chex Plus BC, CD4 Normal designed for Beckman Coulter Q-prep Cytometry system
CD Chex Plus BC, CD4 Low designed for Coulter Q-prep Cytometry system.
Streck CD Chex Plus controls may not be compatible with the BD FACSCount System.

Can you explain the difference between single and dual platforms?

In the CDC MMWR Guidelines for Performing Single Platform Absolute CD4+ T-Cell Determinations (www.cdc.gov/mmwr/preview/mmwrhtml/rr5202a1.htm), the definition of single-platform technology (SPT) is a process in which absolute counts of lymphocyte subsets are measured from a single tube by a single instrument. SPT incorporates internal calibrator beads of known quantity in the analysis of specimens by three- or four-color flow cytometry. Compare this with the definition of a dual platform; dual-platform technology is a method in which absolute counts are derived from measurements obtained from two instruments - a flow cytometer and hematology analyzer.

The FACSCount flow cytometer is a single platform system. The absolute marker values tested in this system may be reported as a percentage of the total lymphocytes with the use of a hematology count for the specimen. For example, a CD4 count of 1218 cells/µl obtained from the FACSCount instrument divided by the total lymphocyte count of 2230 obtained from the hematology analyzer equals a CD4% value of 54.62.

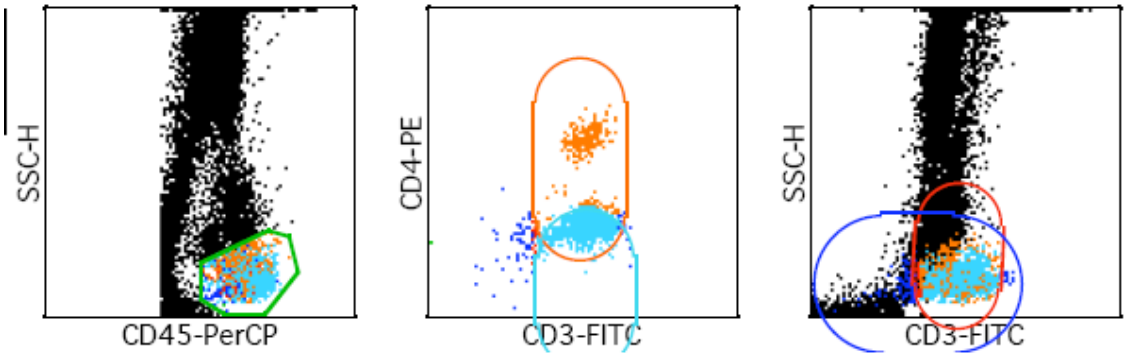
BD has determined the use of a conversion factor to calculate the CD4% values from the CD4/CD3 ratio value obtained in the FACSCount system. To convert the FACSCount data, multiply the CD4/CD3 ratio by 100% and the conversion factor of 0.813. For example; a FACSCount CD4/CD3 ratio of 0.220 is first changed to a percentage by the calculation, 0.220 x 100% = 22.0%, next the % of CD4 of CD3 is

multiplied by the conversion factor, 22.0% x 0.813 = 17.9%, which results in the CD4 percentage value of the total lymphocytes. This conversion calculation formula is limited for use in fresh whole blood specimens from adults and is not applicable for pediatric samples or stabilized whole blood samples such as commercial controls or some proficiency test material.

Can you provide instructions on how to gate the UK NEQAS samples?

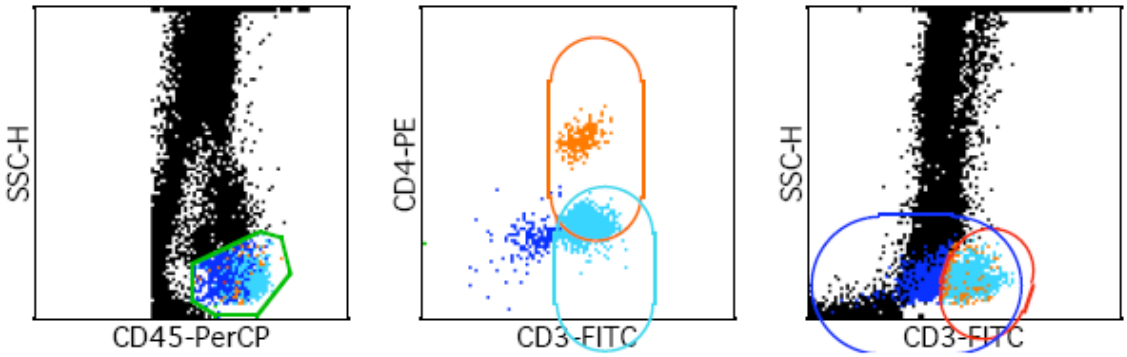
UKNEQAS samples are stabilized whole blood samples with light scatter and fluorescence staining properties that are different from fresh blood specimens and as such may not satisfy the automatic gating algorithm defined by the instrument software. The instrument software may flag the analysis with an error identifying questionable sample integrity. In this instance, a manual gating should be applied to the analysis. The gating of the populations should be reviewed to ensure that the population clusters are gated to include the entire cluster without any interfering background and debris events.

Example of software-defined automatic gating of specimen of *questionable sample integrity*.



QC Messages:
Code 3: Sample integrity questionable. Visually inspect.

Example of manual gating to correct for overlapping interfering events in the analysis.



QC Messages:

Code 7: Manual Gate is in effect.

Can you provide details on what needs to be done to validate a new flow cytometer?

A correlation study using both normal **(non HIV)** and patient samples **(HIV samples)** representing the expected range of reportable result values is recommended to validate a new instrument or method. A minimum of thirty samples should be analyzed on both instruments and correlation statistics applied to determine acceptability of data. In addition, a normal level and low CD4 level should be assayed in replicates (10 times each) on the new instrument to evaluate intra-instrument variations.

What are the IQA's recommendations for the running of calibration beads for clinical labs running CD4 counts?

Clinical labs performing CD4 enumerations should follow manufacturer's recommendations for running daily calibration beads. For the BD FACSCount System, they are the low, medium, and high control beads. For the BD FACSCalibur or FACScan systems, it is the FACSComp Calibrite beads. For the Beckman Coulter Cytometry System, they are the FlowCheck and FlowSet Fluorospheres. In addition, the BD FACSCalibur and Beckman Coulter Cytometer Systems should be monitored monthly for fluorescence linearity. Spherotech Rainbow Beads and ImmunoBrite beads are available to evaluate the linearity of the Becton Dickinson and Beckman Coulter systems respectively.

What are the IQA's recommendations for commercial controls?

Commercial controls are available to monitor the precision and accurately of the testing system. The CD4 normal and CD4 low controls are characterized with expected target

range values. The manufacturer defined ranges have variations greater than 25%. Labs should determine their own target ranges for these control material.

The advantage of using a commercial control is that commercial controls are stable over a period of several months. In addition, the results from the testing of commercial controls can be plotted onto a Levey Jennings chart for monthly monitoring of the system. At the start, a lab should use the manufacturers provided ranges to monitor the daily QC of the lab. As the lab gains more confidence and experience, the ranges provided by the manufacturer should be adjusted. Running commercial controls are not meant to be used to establish the normal ranges.

Does the IQA recommend running replicate samples to check day to day instrument performance? If the answer to this question is yes, do you recommend monitoring % CD4 counts or absolute CD4 counts or both?

It is not necessary to run replicate samples routinely to check day to day instrument performance. The daily control runs would serve as the monitor for instrument and system performance.

Is it necessary to run 2 other controls (? patient bloods) where one has a ratio of <1.0 and the other has a ratio >1.0?

The use of the control beads, low, med, and high beads, serve to monitor the testing system to assess the pipetting accuracy and system linearity. The use of the normal and low level controls serves to monitor the precision and accuracy of the testing protocol. Labs should demonstrate their proficiency to test for values at the levels of routine patient testing. To achieve this, normal level and low CD4 level controls with known target value are recommended. Commercially available controls with normal and low CD4 levels are ready sources for the daily control runs. In addition, a fresh specimen from a normal donor should be included to monitor any variations in testing fresh daily patient specimens.

Patient specimens may be used as a substitute to the commercial controls but are limited to evaluating the target values based on the 1-2 day stability of the sample. A patient specimen that has been tested with a reported CD4 value may be selected as the daily control to monitor the ability of the testing system to replicate this target value. The tolerance limit should be determined based on previous replicate studies performed as part of the instrument validation and set up. The advantage of using a commercial control over a patient sample is that the commercial control is stable over several months. In addition, the results from the testing of commercial controls can be plotted onto a Levey Jennings chart for monthly monitoring of the system. This monitoring approach can not be applied when using a different patient control daily as the target control.

For reagent lot parallel testing, is it necessary to run a patient with a ratio >1.0 and a patient with a ratio <1.0. If so, how reproducible should the results be?

For lot-to-lot reagent comparisons, it is not necessary to run two different levels of samples. A normal sample should suffice and the range of acceptability is the same as that defined in the replicated study performed as part of the instrument validation. Generally, the range of the marker values defined in intra-instrument variation studies are less than 10%.

Other QC measures that you suggest by the IQA

For labs with more than one flow cytometer, a replicate sample should be run daily on each additional instrument in use to show instrument correlation. The tolerance limits of the monitoring parameters should be previously defined by instrument correlation studies.

Full service cytometers should be monitored monthly for instrument linearity. Calibration beads are available to monitor system fluorescence linearity. Beckman Coulter provides ImmunoBrite Beads for this purpose for their cytometry systems. Spherotech Rainbow Beads are available for use in BD cytometry systems.

Procedure: BD FacsCount Procedure for CD4/CD8

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		New

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

Principle:

Absolute CD4, CD8 And CD3 T-lymphocyte counts are used to evaluate the immune status of a patient with or suspected of developing Acquired Immune Deficiency Syndrome (AIDS).

The CD4, CD8, CD3 are surface glycoproteins of T-lymphocytes. The CD4 antigen is the receptor of Human Immunodeficiency Virus (HIV) and this is the cellular parameter mostly associated with HIV disease progression and patient progression to AIDS. In HIV disease there is an imbalance of the CD4 and CD8 lymphocyte ratio due to decline of CD4 T lymphocytes. The enumeration of these cell subtypes is of paramount importance in monitoring infection progression.

The test for enumeration of cells with these surface proteins uses a ready-to–use reagent tube pair. One tube determines the absolute number of helper/inducer T lymphocytes (CD4/CD3) and the other tube determine the absolute number of suppressor/cytotoxic T (CD8/CD3). Both tubes measure the absolute total number of lymphocytes (CD3).

The basis of the assay is that when blood is added to these reagents above, flourochrome labeled antibodies in the reagents bind specifically to lymphocyte surface antigens. After an incubation period a fixative is added and the samples are run on the instrument.

The laser light will cause fluorescing of the flourochrome labeled cells. This fluorescent light provides information necessary for the instrument to count cells. The reagent tubes also contain a known number of flourochrome-integrated reference beads. These beads act as fluorescent standard for locating the lymphocytes and also a quantitative standard for enumerating cells.

The instrument automatically calculates the absolute T lymphocyte populations and the results appear on the screen as well as printed on paper.

Specimen Collection and Handling:

Patient preparation: None

Specimen requirements:

Anticoagulant:	K_3 EDTA tubes
Sample storage:	Keep whole blood at room at room temperature (18 -28^{0}C)
Sample stability:	Sample must be processed within 72 hours of collection.
Sample Volume:	Minimal volume is 150µl
	Nominal volume is 500µl

Unacceptable specimens: Clotted, grossly hemolyzed, grossly lipemic

Equipment Required:

1. BD FacsCount Coring Station – opens reagent/control tubes
2. 50ul precision pipette such as BD FacsCount electric pipette or equivalent – delivers 50µl quantities
3. BD FacsCount software protocol diskette – start up and operates instrument
4. BD FacsCount workstation – holds blood samples, reagent/control tubes and other supplies.
5. Vortex mixer
6. BD FacsCount Instrument

Materials Required:

1. BD FacsCount reagents - Each kit contains enough CD4/CD8/CD3 reagents, caps and fixative solution for 50 tests.
2. BD FacsCount controls – Four bead concentrations (zero, low, medium, high), each kit contains 5 control sets or about 25 control runs.
3. Normal EDTA whole blood –used with the controls
   3.1 For purposes of this procedure a normal EDTA whole blood sample is defined as a sample with a total WBC count of 5000-10,000 and an absolute lymphocyte count of 1000 to 2000.
   3.2 The sample may be diluted to obtain an absolute lymphocyte count with the guidelines given.
   3.3 If a sample is not available within these guidelines, select a sample as close as possible to these guidelines.
4. System fluid – Saline solution such as BD FacsFlow or Coulter Isoton
5. BD Thermal printer paper.
6. Pipette tips to fit 50 ul pipette
7. BD Cleaning Test Tubes with caps (for bleach and distilled water)
8. Test Tubes, 12 x 75 mm (for sample dilutions)
9. Dispensing bottles (for both bleach and distilled water).

Reagent Preparation:

1. BD FacsCount Controls are ready to use. The controls must be brought to room temperature for 30 minutes prior to use. Make sure controls have been stored upright to avoid run problems.
2. BD FacsCount Reagents are ready for use. The reagents must be brought to room temperature for 30 minutes prior to use.
3. Prepare 10% household bleach daily for use in cleaning the instrument.

Storage and Stability:

1. Reagents and controls should be kept at 2 – 8^0c and remain stable until expiration date on kit. Note: Keep controls in upright positions. The reaction beads tend to get stuck in the control cap when the controls are not stored upright which appears to cause control errors.
2. Store blood samples at room temperature and away from direct sunlight. Fresh EDTA whole blood samples are stable for up to 72 hours prior to testing.
3. Processed blood samples can be stored at room temperature away from sunlight and remain stable for a maximum of 72 hours after the addition of the fixative solution.
4. Processed control samples can be stored at room temperature away from sunlight and remain stable for a maximum of 72 hours after the addition of the fixative solution but only 2 hours after the addition of the control beads

Calibration:

1. No calibration required – Pre-calibration is done by the manufacturer.
2. Perform monthly reproducibility checks. Refer to the reproducibility check procedure.

Maintenance:

1. Place the instrument in stand-by during the day when not in use.
2. Clean the instrument daily following the instructions in the shutdown procedure.

Quality Control:

1. The BD FacsCount Controls (zero, low, medium and high) are run daily with the first patient run. These control runs set up the instrument and check the linearity as well as reagent activity.
2. These controls should be run with the first run each day.
3. The control samples must be run and pass before patient samples can be run or reported.
4.

Reference Range:

1. Normal (hematologically) adult (18-65 years) reference ranges for CD4, CD8 absolute counts and CD4/CD8 ratio compiled from three sites (2 United States and one European clinical site) by Becton Dickinson (BD) using the BD FacsCount reagent and instrument.

Sex	Age	CD4ABS cells x 10^9/L	CD8ABS Cells x 10^9/L	CD4/CD8 Ratio
Female	18-65	0.470-1.298		
Male	18-65	0.355-1.213		
Both	18-40		0.208-0.796	
Both	41-65		0.144-0.699	
Both	18-40			0.92-3.41
Both	41-65			0.83-6.10

2. Normal adult (18-65 years) reference ranges for CD4% and CD8% taken from the Textbook of Hematology. Shirlyn B. McKenzie, 2nd Edition, 1996, Table N, Page 702.

   CD4% 35-45%

   CD8% 18-38%

3. Normal pediatric reference ranges for CD4/CD8 absolute counts, percentage and ratio complied from the Harriet Lane Handbook.

Age	CD4ABS Cells x 10^9/L	CD4 %	CD8ABS Cells x 10^9/L	CD8 %	CD4/CD8 Ratio
Birth	0.4-3.5	17-52	0.2-1.9	10-41	1.0-2.6
1wk-2mo	1.7-5.3	41-68	0.4-1.7	9-23	1.3-6.3
2mo-9mo	1.4-5.1	33-58	0.5-2.2	11-26	1.6-3.9
9mo-15mo	1.0-4.6	31-54	0.4-2.1	12.28	1.3-3.9
15-24mo	0.9-5.5	25-50	0.4-2.3	11-32	0.9-3.7
2-5 years	0.5-2.4	23-48	0.3-1.6	14-33	0.9-2.9
5-10 years	0.3-2.0	27-53	0.3-1.8	19-34	0.9-2.6
10-16 yrs	0.4-2.1	25-48	0.2-1.2	9-35	0.9-3.4
16-18 yrs	0.3-1.4	28-57	0.2-0.9	10-39	1.0-3.6

4. Expected normal or reference values may vary depending upon age, sex or race of patient.
5. Adult reference ranges should not be used with pediatric blood samples.

Procedure, B.D FacsCount Controls:

1. Bring BD FacsCount controls and reagents to room temperature for 30 minutes before testing.
2. Fill out the BD FacsCount Reagent Log (copy in appendix) with the lot numbers, expiration dates and assigned control values of the reagents that are being used for the day.
   2.1 Remember to enter the new reagent lot number and run new controls if a new lot of FacsCount reagent is opened during the day.
3. Label the tabs of the two reagent tube pairs as follows:
   3.1 Pair one

   Label CD4 (Green top) – Zero

   Label CD8 (Clean top) – Low

   3.2 Pair two
   Label CD4 (Green top) – Medium

Label CD8 (Clean top) – High

4. Vortex the pair upside down and upright for 5 seconds respectively using a mid-range speed.

5. Open the reagent tubes using a Coring Station.
6. Mix the normal whole blood by inverting the tube five times.
7. Pipette 50µl of well-mixed normal whole blood into each of the 4 reagent tubes above. **Note: Remember to change tips on every pipetting from this stage up to the end.**
8. Cap the tubes and vortex upright for 5 seconds.
9. Incubate the reagent tubes at room temperature for 60 – 120 minutes in the BD FacsCount workstation with a closed cover to protect reagents from sunlight.
10. Uncap the tubes and add 50µl of fixative solution into each tube.
11. Recap the tubes with new caps and vortex upright for 5 seconds. Stained samples may be stored in the workstation for up to 72 hours before adding the control beads.
12. Remove the control reagents from the kit. Leave the controls on the workstation at room temperature for 30 minutes prior to use.
13. Vortex the control upside and upright for 5 seconds respectively. Use the Coring Station to pierce each pair of controls when opened for the first time.
14. Pipette 50µl of the control to each tube recapping the controls with a new cap after use.
    14.1 Pipette 50ul of zero control into the CD4 reagent tube labeled zero.
    14.2 Pipette 50ul of low control into the CD8 reagent tube labeled low.
    14.3 Pipette 50ul of medium control into the CD4 reagent tube labeled medium.
    14.4 Pipette 50ul of high control into the CD8 reagent tube labeled high.
15. Recap the reagent tubes with new caps and vortex the reagent tube upright for 5 seconds.
16. Run the tubes on the FacsCount Instrument within 2 hours of adding the control beads to the reagent tubes starting with the zero control.
17. Prior to running, the control and reagent specific lot codes and bead counts must be entered if new or verified if previously entered.
18. To enter new lot codes or bead counts:
    18.1 Press <CONTROL> from the FacsCount screen to reach the CONTROL BEADS screen.
    18.2 Enter the eight-digit control bead lot code.
    18.3 Press <ENTER> to move to the next field.
    18.4 Enter the bead counts for the Low, Medium and High controls. This information is found on the control bead tray.
    18.5 Press <Confirm> to save this information to the protocol diskette.
    18.6 The REAGENTS screen is then displayed.
    18.7 Enter the eight-digit reagent lot code. This information is found on the foil bag label and on the reagent pair tab. Press <ENTER>.
    18.8 Enter the CD4 and CD8 reference bead counts for the reagent lot. This information is found on the foil bag label.
    18.9 Press <Confirm> to save this information to the protocol diskette.

18.10 Enter the normal control accession number. If the ID number from the previous normal control exists, press <Clear ID> to remove it.

18.11 The FacsCount will now prompt you for the first pair of controls.

19. **CAUTION!** Do not mix different reagent lots when running the controls or samples.
20. To verify current lot codes and bead counts, follow the instructions from #18 above. Press <ENTER> to move to the next field.
    20.1 The values that appear on the screen are those that were entered previously.
    20.2 Verify that these codes and counts match the reagent and controls that you are currently using.
    20.3 Do **NOT** mix different reagent lots when running controls or samples.
    20.4 After entering the normal control accession number the instrument will prompt you for the first pair of controls.
21. Vortex the first reagent pair (CD4-Zero and CD8-Low) upright for 5 seconds.
22. Uncap the CD4-Zero tube (green top) and set the cap aside.
23. Place the reagent pair in the sample holder so the CD4-Zero tube is in the run position.
24. Press <RUN>.
25. When the analysis of the CD4-Zero tube is complete, the sample holder lowers.
26. Remove the reagent pair and recap the CD4-Zero tube.
27. Uncap the CD8-Low tube (clear top) and set the cap aside.
28. Replace the pair so the CD8-Low tube is now in the run position.
29. Press <RUN>.
30. Remove the reagent pair and recap the CD8-Low tube.
31. Follow steps 21 through 30 for the second pair of controls (CD4-Medium and CD8-High)
32. At the end of the control run the FacsCount printout will indicate whether the control run passed or failed.
    32.1 If the run “PASSED”, mark “P” on the control long, check off “Record QC” on the Operational Check Log and proceed with running patient samples.
    32.2 If the run “FAILED”, repeat the run a second time. Mark in comments “Run 2 times”
    32.3 If the control run fails the second time, consult troubleshooting guidelines and contact the lab supervisor for resolution of the problem.
    32.4 Document any problems and corrections on the BD FacsCount corrective action log.
    32.5 Do not run or report any patient samples until the controls have passed.
    32.6 Indicate the QC was completed on the Operational Check Log (copy in appendix) under “Record QC”.
33. Refer to the FacsCount System User’s Guide Manual, Chapter 4, Preparing and Running Controls, pages 39-60 for additional information if needed.

Procedure, Patients Samples:

1. Bring the reagent tube pairs to room temperature for thirty minutes before running.

2. Record the sample information including the absolute lymphocyte value on the CD4CD8 Worksheet. (copy in appendix)
3. Review the absolute lymphocyte count values to determine what dilutions if any are needed.
   3.1 Prepare a dilution of the patient sample with saline (Isoton or FacsFlow) in a small tube using the following guidelines:

<2000 cells/cmm	No dilution	
2000 – 4000	1:2	50 ul patient sample + 50 ul saline
4000 - 8000	1:4	50 ul patient sample + 150 ul saline
>8000	1:6	50 ul patient sample + 250 ul saline

   3.2 Record the dilution used on the worksheet.

4. Label the tab of one reagent tube pair with the patient accession number.
5. Vortex the reagent pair upside down and upright for 5 seconds respectively.
6. Open the reagent pair in the coring station.
7. Mix the patient whole blood by inverting the tube five times.
8. Pipette 50µl of well-mixed patient whole or diluted blood to each tube in the reagent pair. **Note: Change tips between tubes for each sample!**
9. Cap and vortex the tubes upright for 5 seconds.
10. Incubate the reagent tube pair in the workstation for 60 – 120 minutes.
11. Uncap the tubes and pipette 50µl of fixative solution to each tube, changing tips between tubes.
12. Recap with a new cap and vortex upright for 5 seconds.
13. Run the reagent tubes on the FacsCount instrument within 72 hours of addition of the fixative solution.
14. To run a patient sample, press <SAMPLE> from the FacsCount screen or from the CONTROL results screen.
15. Enter or verify the reagent lot code and reference bead counts. Press <Confirm>.
16. Enter the patient accession number.
17. Vortex the reagent pair upright for 5 seconds.
18. Uncap the CD4 tube (green top) and set the cap aside.
19. Place the reagent pair in the sample holder so the CD4 tube is in the run position.
20. Press <RUN>
21. When the sample holder lowers remove the reagent pair and recap the CD4 tube.
22. Mix the reagent pair then uncap the CD8 tube (clear tops) and set the cap aside.
23. Replace the pair so the CD8 tube is now in the run position.
24. Press <RUN>.
25. When the sample holder lowers, remove the reagent pair and recap the CD8 tube.
26. Record the instrument results on a CD4/CD8 result worksheet (copy in appendix).
27. If you encountered any errors while running the sample, press <Continue> to display the sample results. Refer to the printout and Section 7.2 of the BD manual for information on the printed errors.
28. To run the next sample, press <SAMPLE>, enter the patient accession number and follow steps 17 through 26.

29. Do **not** leave the sample probe exposed to air (dry) for extended periods of time. When not running samples place the instrument in standby until the next run.
    29.1Press <UTILITIES>.

    29.2Press <SHUTDOWN>.

    29.3Place a tube of distilled water in tube holder

    29.4Press <RUN>.

30. To start-up from standby:
    30.1Press <STOP>.

    30.2Press <MAIN>.

    30.3Press <SAMPLE>

31. At the end of the day, perform the daily cleaning and shutdown as described in the Shutdown Procedure.
32. Complete the calculations required as described below under 'calculations'.
33. Review and sign your worksheets. Give the completed worksheets to the laboratory supervisor or designee for final technical review.

Calculations:

1. For diluted samples, calculate the absolute CD4 and CD8 counts.
   1.1 Multiply the instrument value by the dilution factor.
   1.2 Example: Absolute lymphocyte count = 4.4
   Instrument value for absolute CD4 = 0.179

   Instrument value for absolute CD8 = 0.548

   1:4 dilution = dilution factor of 4

   Actual absolute CD4 value = 0.179 x 4 = 0.716

   Actual absolute CD8 value = 0.548 x 4 = 2.192

   1.3 Record the undiluted and calculated results on the CD4CD8 Worksheet.

2. Calculate the percent CD4 and CD8 if needed.
   2.1 Divide the abs CD4 result by the absolute lymphocyte count and multiply by 100 to obtain the percent CD4.
   2.2 Divide the abs CD8 result by the absolute lymphocyte count and multiply by 100 to obtain the percent CD8.
   2.3 Example: Absolute CD4 = 0.716
   Absolute CD8 = 2.192

   Absolute Lymphocyte Count = 4.4

   %CD4 = (0.716/4.4) x 100 = 16.3

   %CD8 = (2.192/4.4) x 100 = 49.8

Interpretation of Results:

1. Healthy individuals would be expected to have CD4 and CD8 absolute values within the reference ranges given. In addition, the CD4:CD8 ratio will usually be greater than 1.
2. Individuals with progressing AIDS would be expected to have lower CD4 absolute values, higher CD8 absolute values and a CD4:CD8 ratio of less than 1.
3. For internal checking of results, the CD4% plus the CD8% is expected to be less than 110% total. See below, Unexpected Results for total values greater than 120%.

Unexpected results:

1. Reagent Tube Failure
   1.1 Sometimes a sample may not pass/produce a result.
   1.2 Resolve the status messages and repeat the testing.
   1.3 If repeat testing does not resolve the problem, review the sample results with the laboratory supervisor or designee.
2. CD4% + CD8% = >120%
   2.1 Repeat the CBC to check the absolute lymphocyte value. If this new absolute lymphocyte value corrects this to less than 120%, report the recalculated results along with the new absolute lymphocyte value. Make sure the CBC is corrected for the new absolute lymphocyte and %lymphocyte values as well.
   2.2 If the new absolute lymphocyte value does not correct the total CD4+CD8% to less than 120%, then repeat the CD4CD8 test.
   2.3 If the CD4+CD8%is greater than 120% after re-running, review the results with the LSA or group lead. Report the results with the comment "CD4+CD8>120%,P'seRpt-NewSample".
   2.4 Diluting the sample will sometimes bring the results into a clinically expected range of CD4 + CD8 <120% especially with pediatric samples.

Clinical Application:

Absolute CD4, CD8 And CD3 T-lymphocyte counts are used to evaluate the immune status of a patient with or suspected of developing Acquired Immune Deficiency Syndrome (AIDS).

The CD4antigen is the receptor of Human Immunodeficiency Virus (HIV) and this is the cellular parameter mostly associated with HIV disease progression and patient progression to AIDS. In HIV disease there is an imbalance of the CD4 and CD8 lymphocyte ratio due to decline of CD4 T lymphocytes. Drug interventions in HIV disease have been seen to reverse the above effects thus making monitoring of the CD4 and CD8 lymphocytes parameters necessary.

The clinician/medical officer is responsible for the interpretation of results obtained from this assay. This interpretation is done bearing in mind the clinical manifestations of the patient.

Linear Range:

1. Results are linear for CD4 from 1-2000 CD4+cells/uL.
2. Results are linear for CD8 from 1-2000 CD8+cells/uL.
3. The CD4 and CD8 absolute counts vote out at values greater than 2000 cells/mm^3.
   3.1 Whole blood samples with total lymphocytes counts greater than 2000 cells/mm^3 require dilution.
   3.2 The diluted result is then multiplied by the dilution factor to obtain the final result (refer to patient sample procedure guidelines, section 3).

Limitations of Procedure:

1. Sample processing must be done away from direct sunlight.
2. Blood should not be stored longer than 72 hrs prior to preparing.
3. Samples with lymphocyte counts > 2000 cell/mm^3 need dilution before running.
4. A CBC must be run to obtain the absolute lymphocyte count for determining the dilution needed as well as calculating the CD4 and CD8% values. Absolute lymphocyte values from other labs will be accepted if the value is obtained on the same sample.
5. The sample probe occasionally blocks. Follow the guidelines in the BD FacsCount Manual for unblocking.
6. Nucleated Red Blood Cells may affect the absolute lymphocyte count. Follow the procedure for re-calculating the total WBC and absolute lymphocyte count found in the Hematology manual. Use the corrected absolute lymphocyte count to calculate the CD4 and CD8%.

Procedural Notes:

1. Any troubleshooting with the instrument is indicated as status messages on the screen. These are printed at the end of the run. (See BD FacsCount Manual, chapter 7). Proceed according to list of possible causes and solutions.
2. Document any problems and troubleshooting on the BD FacsCount Corrective Action Log for each instrument.
3. If you are unable to resolve the instrument problem, contact the LSA or Group Lead for direction.

4. If engineering service is needed for the instrument, contact local BD service representative:
5. If the instrument will not be serviced and operational within 24 hours, send the CD4/CD8 samples to another lab if possible. Notify the clinic immediately.
6. Ideally samples will be run within 48 hours of collection. If this is not possible, the samples can be run within 72 hours of collection per recent studies by Becton Dickinson.

References:

1. BD FacsCount System User's Guide (Feb 1995).
2. The Harriet Lane Handbook, The Johns Hopkins Hospital, fifteenth edition, George K. Siberry and Robert Iannone, chapter 16, page 341, table 16.6, for Pediatric CD4/CD8 reference ranges.
3. Textbook of Hematology, Shirlyn B. McKenzie, 2nd Edition, 1996, Table N, page 702.
4. Technical Assistance E-mail from BD, East Africa Unit, regarding sample storage and stability, 30Sep00.

Procedure: OPERATION of the BD FACSCOUNT ELECTRONIC PIPETTE

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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Principle:

The BD FacsCount electronic pipette operates by reverse pipetting. It is preprogrammed to operate in the reverse pipetting mode to consistently deliver 50 uL of fluid. The preprogramming eliminates the need to make program adjustments using the keypad. Therefore, the keypad on the pipette is inoperable.

Materials Required:

1. BD FacsCount Electronic Pipette and charging unit
2. Pipette tips suitable for 50 uL pipette (standard yellow tip or equivalent)

Calibration:

1. Follow the pipette calibration schedule outlined in the General Laboratory Polices and Procedure Manual.
2. Re-calibrate per the outlined schedule or more frequently if a problem is suspected.

Procedure:

1. The arrow on the pipette display indicates the direction of the fluid when you press the 'Start' button.
   1.1 When the arrow points up toward the top of the pipette, pressing the 'Start' button fills the tip.
   1.2 When the arrow points down toward the tip, pressing the 'Start' button dispenses liquid.
2. Place a clean tip on the pipette.
3. Place the tip 2 to 4 mm into the liquid.
4. Press the pipette 'Start' button.
   4.1 Quickly release the button after pressing.
   4.2 Keep the tip in the liquid pausing 2 to 2 seconds to allow the correct volume of liquid to fill the tip.
   4.3 Touch the tip to the inside surface of the vessel to remove the excess liquid from the outside of the tip **before** dispensing into the reagent tube.
5. Dispense the liquid by placing the tip against the wall of the reagent tube just **above** the liquid level and pressing the 'Start' button.
   5.1 Pause 1 to 2 seconds to allow the correct volume of liquid to enter the reagent tube.
   5.2 Some liquid will remain in the tip.
6. Remove the tip from the reagent tube.
7. Place the pipette and tip over an appropriate biohazard container and squeeze the tip eject lever.
8. Continue with step 2 to pipette additional samples.

Linear Range:

1. A properly calibrated BD FacsCount Electronic pipette should provide between 49-51 uL.

Procedural Notes:

If the BD FacsCount controls are out of range perform the following steps to determine if the problem may be related to the pipette.

1. Run controls.
2. Press the Service Button 10 times.
3. Look through the information presented for the slope, intercept and R value.
4. The slope should be between 0.7 to 1.3.

Slopes higher or lower than this range may indicate problems with the pipette

References:

1. BD FacsCount User's Guide, February 1995, Appendix A, pages 141-149.

Procedure: BD FACSCOUNT REPRODUCIBILITY CHECK

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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Principle:

Monitoring the within-sample reproducibility provides assurance that the BD FacsCount instrument is performing within the guidelines expected by the manufacturer. Within-sample reproducibility checks can also provide an early detection of potential instrument problems such as partial sample probe blockage. If the reproducibility checks exceed the manufacturer guidelines early troubleshooting may prevent instrument down time.

Specimen Collection and Handling:

1. Fresh patient specimens are used for the reproducibility check.
2. The specimens are handled and processed the same as a patient sample.

Procedure:

1. The reproducibility check is performed once per month.
2. If reproducibility problems are suspected the reproducibility check can be performed as needed.

3. Prepare and run patient samples as outlined in the 'BD FacsCount Procedure for CD4/CD8'.
4. Select a patient sample with a CD4/CD8 ratio >1.5 and at least 100 CD4+ and CD8+ cells/uL.
5. Run this sample an additional 3 times on the same instrument for a total of 4 runs.
6. Select a second random patient sample with a CD4/CD8 ration of <1.0 and at least 100 CD4+ and CD8+ cells/uL.
7. Run this sample an additional 3 times for a total of 4 runs.
8. Complete the Reproducibility Check Log.
   8.1 If the difference between the highest and lowest sample values are within the reproducibility guidelines listed, sign the Check Log and submit to the laboratory supervisor for review.
   8.2 If the difference between the highest and lowest sample values are greater than the reproducibility guidelines listed, review the results with the laboratory supervisor for further action.

Procedural Notes:

1. The guidelines for evaluating the within-sample reproducibility have been set at approximately 1.5 SD of the manufacturers listed reproducibility.

References:

1. BD FacsCount User's Guide, February 1995, Appendices Section D.1 Performance Characteristics, Within-Sample Reproducibility.

Procedure: BD FACSCOUNT DAILY START-UP

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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Principle: Daily start up is performed on the BD Facscount instrument on a routine basis. The start up procedure is done by the Facscount software protocol diskette.

Equipment and Materials:
1. B.D FacsCount Instrument
2. Protocol Diskette

Reagents:
1. Bleach or equivalent decontaminating fluid.
2. System Fluid (saline such as BD FacsFlow or Coulter Isoton or equivalent)

Procedure:

1. Check the fluidics system to make sure the fluid reservoir is filled.
   1.1 Open the fluidics compartment door to access the system fluid and waste reservoirs.
   1.2 Disconnect the system fluid tubing from the fluidics panel.
   1.3 Disconnect the system fluid detection probe connector from the fluidics panel.
   1.4 Remove the system fluid reservoir from the instrument and stand it on end before removing the cap.
   1.5 Fill the reservoir with system fluid.
   1.6 Recap the reservoir and replace it in the instrument.
   1.7 Reconnect the system fluid tubing and fluid detection probe connector.
   1.8 Check to see that the fluid filter is filled with fluid.
2. Check that the waste reservoir is empty except for the decontaminating fluid (about 200mls of undiluted household bleach).
   2.1 Disconnect the waste tubing from the fluidics panel.
   2.2 Disconnect the waste fluid detection probe connector from the fluidics panel.
   2.3 Remove the waste reservoir from the instrument and stand it on end.
   2.4 Empty the reservoir.
   2.5 Add 200 ml of undiluted bleach to the empty reservoir.
   2.6 Recap the reservoir and replace it in the instrument.
   2.7 Reconnect the waste tubing and fluid detection probe connector.
3. Make sure the protocol diskette is in the floppy drive.
4. Turn on the power to the FACScount instrument.
5. Allow the system to start up.
   5.1 This is an automatic process carried out by the protocol diskette.
   5.2 After about two minutes, status messages will show on the FACScount screen.
6. Check the flow cell for air bubbles each day after you turn on the instrument. Prime the system if air bubbles are visible.
   6.1 Press <UTILITY> from the FacsCount screen to display the UTILITY screen.
   6.2 Press <DRAIN>.
   6.3 Press <DRAIN> again.
   6.4 When all the fluid has drained from the flow cell, press <STOP>.
   6.5 Press <MAIN> to return to the FacsCount Screen.
   6.6 Refer to the BD FacsCount Users Manual, section 3.2.3, pages 30-32 for additional information if needed.
7. Check the Date and Time. If incorrect, reset as follows:
   7.1 Press <SetDate> from the SETUP screen.
   7.2 Enter the new date using the numeric keypad. Press <ENTER> after inputting the value for each field.
   7.3 Enter the new time pressing <ENTER> after inputting the value for each field.
   7.4 Press <Confirm> to save this information to the protocol diskette and return to the SETUP screen.
8. Leave instrument to warm up for 15 minutes before running controls or samples.
9. The instrument is now ready for sample and control running.
10. Record start up on the ‘Operational Check Log’.

Procedural Notes

1. A back-up copy of the protocol diskette is stored in the laboratory software library.
2. **WARNING:** Do not dispose of waste reservoir contents until at least 30 minutes after the completion of the last run. This helps to inactivate any biohazardous materials before disposal. A spare waste reservoir is provide, allowing you to continue with the run while waiting 30 minutes before emptying the full reservoir.

Reference: The Becton Dickinson Immunocytometry Systems Manual, February 1995.

Procedure: B.D FACSCOUNT CLEANING and SHUTDOWN PROCEDURE

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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Principle:
Daily cleaning of the BD FacsCount instrument helps ensure instrument reliability and performance. The cleaning is done at the end of each day, when a screen message appears recommending the cleaning cycle be run or when recommended in the troubleshooting section.

The shutdown procedure is performed at the end of each day. Proper shutdown allows for proper storage of the instrument probe while not in use overnight.

Equipment and Materials:
BD FacsCount Instrument
Distilled water
1:3 dilution of bleach
10% household bleach
BD Cleaning Test Tubes with Caps

Procedure:
1. Fill one cleaning tube with a 1:3 dilution of bleach and a second cleaning tube with distilled water.

2. Press <UTILITY> from the FacsCount screen to display the UTILITY screen.
3. Press <CLEAN>.
4. Place the tube of 1:3 bleach solution in the sample holder and press <RUN>.
5. When the sample holder lowers place a cleaning tube with distilled water in the sample holder and press <RUN>. This rinses the bleach solution.
6. To continue running samples, press <MAIN>.
7. To continue with shutdown, press <SHUTDOWN> from the UTILITY screen.
8. Place a tube of distilled water in the sample holder and press <RUN>.
9. Turn off the power supply to the instrument.
10. Complete the department cleaning by:
    - 10.1 Wiping the pipette with a soft cloth dampened with 10% bleach followed by distilled water.
    - 10.2 Wiping the BD FacsCount instrument with a soft cloth dampened with 10% bleach followed by distilled water.
    - 10.3 Wiping the workstation with a soft cloth dampened with 10% bleach followed by distilled water.
    - 10.4 Flush the coring station with tap water to remove any proteins that may have accumulated on the cutters by inverting the coring station under running water. Allow the water to run into the two metal openings surrounding the cutters. Shake out the excess water and dry with a clean dry cloth.
    - 10.5 Wiping the workbench with a soft cloth dampened with 10% bleach.
11. Complete the Operational Check Log marking off the shutdown and cleaning.

Reference:

BD FacsCount Users Manual, February 1999.

BD FACSCOUNT, OPERATIONAL CHECK LOG for Month_____Year_____

Date	Discard 4 day Samples	Start Up	Record Reagents Run QC	Shut Down	Clean & Charge Pipette	Clean w/ 10% Bleach/Jik				Reprod Check Monthly	QA Project Monthly	Tech Initials	Comments
						FacsCount Instrument	Work Station	Coring Station	Work Bench				
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													
13													
14													
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Reviewed by:_____ Date:_____

BD FACSCOUNT REAGENT LOG

Date	Reagent Lot#	Reagent ExpDate	Control Lot#	Control ExpDat	Control Count, beads/ml				Contr P/F	Saline Lot#	Saline ExpDate	Comments	Tech Initials
					Zero	Low	Med	High					

Procedure: BD FACSCOUNT INSTRUMENT INFORMATION

Prepared by	Date Adopted	Supersedes Procedure #

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

If the BD FacsCount controls are out of range perform the following steps to determine if the problem may be related to the pipette.

1. Run controls.
2. Press the Service Button 10 times.
3. Look through the information presented for the slope, intercept and R value.
4. The slope should be between 0.7 to 1.3.
5. Slopes higher or lower than this range may indicate problems with the pipette.

Note: 90% of all BD FacsCount problems are related to cleaning. Make sure the instrument is cleaned routinely. Check the probe for blockage. Blockage may be partial which will give random results, low laser power messages and a variety of other syptoms.

The above information was received from Nick Bright, BD Technical Services, during a routine visit on 4 January 2001

If a pipette problem is suspected, repeat the controls using the current pipette and a newly serviced pipette. Compare the results. If the newly service pipette results are within range and the current pipette results are not, place the new pipette into service and return the current pipette for service/calibration.

Probe Blockage – no activity detected by the instrument.

If the probe appears blocked:

1. clean with bleach
2. clear with BD stylets
3. replace with new probe if necessary (notify supervisor before changing)

Note: The probe may be pushed against the reagent tube or beads in such a manner that the sample can not be aspirated. To check for mis-alignment, try aspirating distilled water from a cleaning tube. If the water aspirates the probe is not blocked but is mis-aligned.

CD4/CD8 WORKSHEET – BD FACSCOUNT

ACC#_____ STUDY CODE/ID#_____ SPECIMEN DATE_____

CBC: LYMPH ABS_____Dilution_____ RUN BY_____RUN DATE_____

CD4%_____ CD4ABS_____ CD4/CD8 RATIO_____ Repeat #_____

CD8%_____ CD8ABS_____ RUN BY_____ RUN DATE_____

COMMENTS: ____CD4+8>120%,P'seRpt-NewSample _____ Test Not Reportable, Please Submit New Sample _____ Other,

Section 14: HIV Testing

Rapid Tests, EIA and Western Blot

1. Overview: Considering that HIV status is the primary endpoint in many HPTN/MTN studies and the medical and social consequences of a person's HIV status, the importance of reporting accurate and timely HIV results cannot be overstated.

   Every study that performs HIV testing will have a specific testing algorithm which is stepwise tests used to determine HIV status. This algorithm must be validated locally at each site and approved (guidelines follow). All sites will need to be aware of local regulations (i.e. two rapid tests, certified technologists and /or VCT counselors) which may apply. Some studies will require that FDA approved tests be used. Some tests may be available in FDA approved and Non-FDA approved versions and sites are responsible to make sure they are purchasing FDA kits when required.

   Rapid tests are frequently used in screenings and the first step in an algorithm. The advantages of rapid tests are that they are fast, easy, inexpensive and generally don't require a lot of equipment. Frequently a combination of HIV rapid tests will be used to improve sensitivity and specificity.

   ELISA or EIA tests may also be used in screening and routine testing. They are expected to have better sensitivity and specificity but may cost more, require a higher level of technician expertise and take longer and often need to be done in batches. The Western Blot test is used only as a confirmation for specimens already tested by rapid or ELISA.

2. Specimen: most rapid testing will be done on whole blood, anticoagulated blood, plasma or serum. The lab should validate the specimen type that will be used at their site. The ELISA or Western Blot assays allow for serum or plasma.

3. Internal Quality Control: Each rapid test has a built in internal control. In addition to the internal control, external controls should be tested by new testing personnel, new lot, new control lot, at a minimum weekly, and whenever a temperature fluctuation has occurred. For the ELISA test, a minimum of 3 negative controls and 2 positive controls should be assayed with each run. An additional external low reactive control should be included. A high reactive, weak reactive and negative control must be included in each Western Blot run.

4. External Quality Control: HPTN/MTN sites must perform the appropriate CAP basic rapid test panels and/or Viral Marker panel. Additional external QC may be performed as desired.

5. Calibration: N/A

6. Specimen analysis: When running two rapid tests, discordant results may occur. In these cases, careful review of the results and additional confirmatory testing is required. Studies have shown that some of these patients may be acute sero-converters. Most studies will have a WB performed to confirm the diagnosis. In the case of sero-converters, some studies will have a HIV-1 RNA PCR performed to confirm diagnosis.

   In some of the HPTN trials, it is a requirement to have 2 readers of the rapid tests. In the case of reactive results, the second reader may need to be a clinician. The readers need to be trained and each kit must be read by both within the time frame of the assay.

When performing EIAs, typically the reactive samples will be run in duplicate prior to performing confirmatory testing. If two out of the three samples are reactive, the patient is considered reactive and sent for confirmatory testing. If the ODs are around the cut-off or <1.0 signal to cut-off ratio, the patient may be cross-reacting and the WB pattern will be indeterminate.

7. Microscopy: N/A

8. Troubleshooting specimens: When there is a noticeable increase in discordant rapid tests, the HPTN/MTN NL should be notified.

9. Maintenance:
    a. The rapid test kits require minimal instrumentation. Timers and temperature checks (including room temperature) are required.
    b. The ELISA tests require more equipment than the rapid tests and therefore, more maintenance. Besides checking the timers and temperatures, the pipettors, plate washer and plate reader all require general maintenance on a daily, weekly, monthly, semi-annual and annual basis.
    c. The Western Blot procedure may be done with automated instrumentation, which requires maintenance to be performed or performed manually. The manual method requires additional checks: RPM on the rotator, cleaning/disinfecting the aspirator system.

HIV PCR/Resistance testing

1. Overview: Some protocols need to quantify the amount of HIV present in infected individuals. This is done through HIV RNA viral load testing using the Polymerase Chain Reaction (PCR). Other protocols may rely on the HIV DNA qualitative assay using PCR to determine HIV status in infants. Also of potential importance is anti-viral resistance testing, typically done through genotyping. These methods require highly trained technicians and are expensive. A cross network effort has allowed technology transfer of the ViroSeq assay to several international sites, including labs in Brazil, South Africa and India. ViroSeq information is now on Abbott Molecular Diagnostics web site:

   http://www.abbottmolecular.com/InfectiousDiseases_1246.asp

2. Specimen: HIV RNA PCR and genotyping assays require the use of EDTA or ACD (note: dilution factor involved) plasma that has been processed within 6 hours of collection. Studies have shown that samples for viral load can be held out to 48 hours before processing. The HPTN/MTN encourages processing within the 6 hour time frame but realizes that this may not always be possible. Any delayed processing should be indicated in the specimen tracking logs. HIV DNA PCR requires the use of cells isolated either by ficoll hypaque gradient centrifugation or by the lysing of the red blood cells with a lysing reagent provided in the Roche kit.

3. Internal quality Control:
    a. The HIV RNA PCR test requires that a negative, low positive and high positive control be assayed with each run.
    b. The HIV DNA PCR assay requires the use of VQA blinded controls plus VQA log standards. Kit controls should be also be assayed along with the VQA controls.

4. External Quality Control: HPTN/MTN sites must perform the appropriate VQA panel.
    a. Each site must pass a 20 member certifying panel and two 5 member panels prior to testing participant samples for HIV RNA PCR.
    b. Each site must pass a 30 member cell pellet qualifying panel plus at a minimum one real time panel.
    c. Two VQA panels and one Abbott panel must be passed to become certified for genotypic resistance testing.

5. Calibration: Will vary based on the equipment in use.

6. Microscopy: N/A

7. Troubleshooting Specimens: Heparin or serum samples are not appropriate for PCR testing. If a heparin sample is obtained, a special extraction procedure is required.

8. Maintenance: In addition to the maintenance required for ELISA assays, the PCR assays involve maintenance of the specialized equipment including thermocyclers, Cobas and sequencing instruments.

HIV Algorithm Validation Requirements

HPTN research sites performing HIV-1 antibody testing for HPTN protocols must validate each HIV antibody testing algorithm that they intend to use for any HPTN study in which incident HIV infection is the primary endpoint. FDA approved HIV antibody tests are recommended especially for HPTN protocols conducted under an IND, but regardless of which HIV antibody tests are used, each site needs to validate the sensitivity and specificity of the testing algorithm they will use for HPTN protocols. This validation is for the algorithm, so if the same algorithm is used for multiple protocols, the validation need not be repeated for each study. If different algorithms will be used for different protocols, each algorithm needs to be validated.

The validation process requires testing specimens from 100 confirmed HIV-infected individuals and 100 confirmed HIV-uninfected individuals using an FDA approved kit along with the kit (or kits) planned for use in a study. If serum or whole blood (venous) will be used at the site for future testing then serum or whole blood specimens must be tested. If whole blood finger prick specimens will be tested, then the assays will need to be performed using finger prick specimens obtained from confirmed HIV infected and uninfected subjects. The person testing the specimens for validation should be blinded as to the HIV status of the specimens.

For example, if both the Abbott Determine and Ora-Quick tests will be used on whole blood with resolution of discrepant results by HIV-1 Western blot, then at least 100 HIV positive samples and 100 HIV negative samples should be obtained, for which the positives have been confirmed positive by Western blot or IFA that is approved by the FDA or your country's regulatory authority. The negatives should be confirmed negative by an FDA approved HIV EIA or by two FDA approved rapid HIV tests. The final result of the testing algorithm should then be compared to the positive or negative result of the reference test (i.e., Western blot if positive or EIA if negative).

If testing reveals one or fewer false-positive or false-negative results, then the testing algorithm has been validated and the testing algorithm is approved for use in HPTN protocols. If testing results in more than one false-negative or false-positive sample, then more specimens will need to be tested.

If it is later decided to use a different algorithm for a study at the site, the validation process should be repeated, as described, for that algorithm.

Each site should send a validation report to Estelle Piwowar-Manning (epiwowa@jhmi.edu) describing the validation process used and the results of the testing in terms of sensitivity and specificity. Upon review of this documentation, the CL will provide written concurrence that the algorithm has been approved for use in HPTN studies.

There may be unique circumstances at each site that require clarification or modification of this validation process. Sites are encouraged to contact the CL for further guidance and to provide the CL with the plan for completing this requirement in advance of implementation to ensure that the process to be undertaken is adequate If subjects have given consent to be tested for HIV, additional consent should not be necessary. Since this is a quality assurance activity, not a research activity, IRB review is not required, at least in the United States.

Procedure: ABBOTT DETERMINE HIV 1 AND 2 RAPID TEST

Prepared by	Date Adopted	Supersedes Procedure #
Constance Ducar		

Review Date	Revision Date	Signature

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Principle:
The Abbott determine HIV1/2 is an in vitro visually read immunochromatographic assay for the qualitative detection of antibodies to HIV-1 and HIV-2 in human serum, plasma or whole blood.

Sample is added to the sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are present in the sample, the antibodies bind to the antigen-selenium colloid and to the antigen at the patient window, forming a red line at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are absent, the antigen- selenium colloid flows past the patient window site. To ensure assay validity, a procedural control bar is incorporated in the assay device. The test is invalid if the control bar does not appear.

Specimen collection and handling:
1. Serum, EDTA plasma or EDTA whole blood collected by venipuncture; should be collected aseptically to avoid hemolysis. Use EDTA collection tubes for plasma and whole blood specimen.

Specimen Storage:
1. Serum, plasma or whole blood may be stored at room temperature (15-30° C) for up to 24 hours.
2. Serum and plasma specimens may stored at 2-8°C for up to 7 days.
3. Serum and plasma may be stored frozen (20°C or colder) indefinitely.
4. Whole blood collected by venipuncture may be stored at 2-8°C for up to 7 days. Do not freeze whole blood specimen.

Sample Requirement:
1. Minimum sample volume required is 50µl; ideal sample is volume 200µl.
2. Unacceptable specimens include:
   2.1 Hemolyzed specimens (interferes with reading patient and control bars)
   2.2 Specimens stored at 15-30° C for more than 24 hours.
   2.3 Specimen stored at 2-8° C for more than 7 days.

Materials Required:

1. Abbott Determine HIV 1 / 2 test strips (100 test strips/kit, 10 test strips/card x 10 cards)
2. Bottle of 2.5ml chase buffer for whole blood samples.
3. Positive and Negative controls (per each new lot).

Equipment Required:
1. Pipettes 50µl precision pipette.
2. Pipette tips

Reagent Preparation: None

Storage and Stability: Store Determine kits and chase buffer at 2-30° C until the expiration date listed on the kit.

Calibration: None

Quality Control:

1. External controls are run with each new kit lot.
   1.1 For HIV-1, run a positive control for HIV-1 and a negative control.
   1.2 For HIV-1 & 2, run a positive control for HIV-1 and 2 and a negative control.
   1.3 The control bar must show clearly for all controls.
   1.4 Negative control should not show any band at the patient window.
   1.5 Positive control should show bands clearly on the control window and patient window.
   1.6 Record the control results along with lot numbers and expiration dates on the Determine control log.
   1.7 If controls fail to give the expected results, repeat the testing. If results are still out of control, consult the lab supervisor and record the problem on the Determine corrective action log. Do **NOT** run patient samples until the control problem has been resolved.

2. To ensure validity of the assay, a procedural control is incorporated in the device and is labeled "CONTROL". If the control bar does not turn red by assay completion, the test result is invalid and the sample should be re-tested.

Test Procedure:
1. Always label the test strips correctly before performing the test.
2. Do not touch the test strip once it has been opened.
3. Opened strips should be used immediately.
4. Reseal all unused test strips in the foil pouch containing the desiccant.
5. Remove the desired number of test units' from the 10 test cards if not using the whole 10-test card by bending and tearing at the perforation.
6. Remove the protective foil cover from each test strip.
7. For serum or plasma samples:
   7.1 Apply 50µl of sample (precision pipette) to the sample pad (marked by the arrow symbol).

7.2 Read at 15 minutes and record results on HIVSCR worksheet.

8. For venipuncture whole blood sample:
   8.1 Mix sample well
   8.2 Apply 50µl of sample (precision pipette) to the sample pad (marked by the arrow symbol).
   8.3 Wait a minute, then apply one drop of chase buffer to the sample pad.
   8.4 Read at 15 minutes and record results on HIVSCR worksheet.
   8.5 Do not read past 15 minutes; erroneous results may occur.

Calculations: None

Interpretation of Results:

The strip should be carefully inspected for the presence of red bar at the control window labeled "CONTROL" and the patient window labeled "PATIENT" on the test strip.

1. Positive (two red bars)
   1.1 Red bars appear in both the control window (labeled 'control') and the patient window (labeled 'patient') of the test strip.
   1.2 Any visual red color in the patient window should be interpreted as "POSITIVE".

2. Negative (one red bar)
   2.1 One red bar appears in the control window of the strip (labeled "Control") and no red bar appears in the patient window of the strip (labeled "Patient").
   2.2 Report as "NEGATIVE"

3. Invalid (No Bar)
   3.1 If there is no red bar in the control window of the strip and even if a red bar appear in the patient window of the strip, the result is invalid.
   3.2 Repeat the test.
   3.3 If the sample still gives an invalid result, report as "INVALID". Run the patient sample by another method.
   3.4 Review all results from an invalid strip with the laboratory supervisor before reporting.

Unexpected Results:

1. Occasionally test strips may show a very weak or broken control band. If the control band is very weak, repeat the Determine test. If the control band is still very weak, run the patient sample by another method to confirm the results.

Result Reporting:

1. The Determine test is reported as Positive, Negative or Invalid as the bars indicate.
2. All Determine "NEGATIVE" results with valid control bars may be released.
3. All Determine "POSITIVE" results must be verified by a second method.
4. All Determine "INVALID" results must be run by a second method.
5. Refer to the current HIVSCR algorithm for final interpretation of result.

Clinical Application:

1. Determine HIV 1 / 2 is a sensitive screening test to aid to determining HIV 1/ 2 infection.
2. Determine HIV 1 /2 should be used as part of an algorithm to increase the accuracy of results.
3. A slight false positive rate has been seen during test evaluation.

Linear Range: None

Limitation of the Procedure:

1. The Abbott determine HIV1/ 2 test is designed to detect antibodies to HIV 1/ 2 in human serum, plasma and whole blood. Other body fluid or pooled specimen may not give accurate results.
2. The intensity of the patient bar does not necessarily correlate to the titer of antibody in the specimen.
3. No test provides absolute assurance that a sample does not contain low levels of antibodies to HIV –1 or HIV – 2 such as those present at a every early stage of infection. Therefore a negative result at any time does not preclude the possibility of exposure to or infection with HIV –1 or HIV – 2 viruses.
4. Positive specimens should be re-tested using another method and the results should be evaluated in light of the overall clinical evaluation before diagnosis is made. (Refer to HIVSCR algorithm).
5. Whole blood or plasma specimens containing anticoagulants other than EDTA may give incorrect results.
6. Grossly Hemolyzed specimens are not suitable for testing due to the background they give on the test strip. Therefore it is not possible to establish results on a Hemolyzed sample.

Procedure Notes (Trouble shooting):

1. Wear personal protective equipment and gloves.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where these materials are handled.
4. Clean and disinfect all spills of specimens or reagents using a suitable disinfectant such as 10% bleach.
5. Decontaminate and dispose of all specimens, reagents and other potentially contaminated materials in accordance with local bio safety regulations.
6. Red spots at the patient window sides may give the erroneous impression of a positive band when using whole blood specimen. If this is suspected, repeat the test using serum or plasma.
7. Results from test strips with invalid control bars must NOT be reported.
8. Very weak control bars may indicate a problem. Repeat the test or un the patient sample by another method to verify.

References:

Determine HIV 1/ 2 insert.

ABBOTT DETERMINE CONTROL LOG

Date	Determine Kit Lot # / Exp Date	Negative Control				HIV Positive Control				Comments Initials
		Manufact	Lot/Acc #	Exp Date	Rapid Result	Manufact	Lot/Acc#	Exp Date	Rapid Result	

QC must be performed with each new lot. Controls may be run more frequently if problems are suspected or unusual results are encountered.

YRG CARE Infectious Diseases Laboratory

Vironostika HIV Uni-FormII Ag/Ab		
SOP Number: Immunochemistry\004\Ver 3.0\2005	Total Number of Pages: 9	
Primary Author: H. SYED IQBAL	Signature & Date:	
Reviewed by: H. SYED IQBAL	Signature & Date:	
Revision History: 1st Revision – Jan 2003, 2nd Revision – Jan 2004, 2nd Revision – Jan 2005		
Distributed to: Immunochemistry Lab and copy to Library		
Approved by: Dr. K.G.Murugavel	Signature & Date:	
Distribution Date:	Effective Date:	Revision Due Date:

SCOPE : This SOP has been prepared for the HIV ELISA assays. The Technologists in Immunochemistry will do the investigations.

PURPOSE: To ensure the quality testing and to provide reliable results.

PRINCIPLE:

Vironostica HIV Uni-FormII Ag/Ab is an ELISA based on a one-step "sandwich" principle and which detects antibodies to HIV type 1 and /or 2(anti-HIV-1,anti HIV-2 and anti-HIV-1 group O) and HIV-1 antigen in human serum or plasma. Micro wells are coated with HIV-1 gp160, HIV-1 peptide, HIV-2 env peptide (amino acids 592-603) and anti-HIV-1 p24.Each micro well contains an HRP – labeled conjugate sphere of the same HIV-antibody / antigen mixture. The specimen diluent, which is added to the wells first, will dissolve the conjugate sphere. Then the test sample or appropriate control containing HIV antibody or antigen is incubated in the micro wells.

If HIV-1 and /or HIV-2 antibody is present in the sample, a solid phase antigen / anti –HIV/-enzyme labeled antigen complex is formed. If HIV-1 antigen is present in the sample, a solid phase antibody /HIV antigen /enzyme labeled antibody complex is formed. Following a wash

procedure an incubation with TMB substrate, color develops which turns yellow when the reaction is stopped with sulfuric acid. If an anti-HIV-1, anti –HIV-1/2, anti-HIV-1group O and /or HIV antigen is present in the sample, an intense color develops. However, when the sample is free of anti-HIV and HIV antigen, no or low color forms with the addition of substrate.

SPECIMEN : Plasma or Serum may be used.

Sample handling condition:

Short storage - Sample can be stored at 2-8^{o}C for unto 1 week
Long storage - Sample can be preferably stored frozen at -20^{o}C

REAGENTS:

REAGENTS	CONTENT	PRESERVATIVE
MicroELISA strips(Breakable)	HIV-1 gp160 HIV-1 ANT70 HIV-2 env(aa 592-603)	--
Negative control	Normal Human serum	0.1g/l gentamycin sulfate 0.2ml/l cinnamaldehyde
Anti-HIV-1 Positive Control	Human monoclonal anti-HIV-1	0.1g/l gentamycin sulfate 0.2ml/l cinnamaldehyde
Anti-HIV-2 Positive Control	Human monoclonal anti-HIV-2	0.1g/l gentamycin sulfate 0.2ml/l cinnamaldehyde
HIV-1 Antigen Positive Control	HIV-1 p24 (inactivated)	0.1g/l gentamycin sulfate 0.2ml/l cinnamaldehyde
Specimen Diluent	Stabilizing protein & Detergents	0.2% Kathon CG
TMB Solution	Tetramethyl benzidine in Citric acid	1 g/l 2-Chloroacetamide
Urea peroxide solution		1 g/l 2-Chloroacetamide
Wash buffer	Phophate buffer	

REAGENT PREPARATION : Before using or reconstituting bring the reagents to reach room temperature.

Wash Buffer : Concentration of wash buffer is 25x. Dilute the Phosphate buffer in the ratio of 1:25 with deionized / distilled water.

TMB Substrate : TMB solution and Urea peroxide should be combined in an equal volume.
Stopping solution (H_2SO_4) : For 1 litre, 50ml conc. acid (18 mol/l) to 850ml of deionized water.

REAGENT REQUIREMENTS:

REAGENT	**NO. OF STRIPS**											
	1	2	3	4	5	6	7	8	9	10	11	12
SAMPLE DILUENT **(in µl)**	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB + UP **(in µl)**	0.5 + 0.5	1.0 + 1.0	1.5 + 1.5	2.0 + 2.0	2.5 + 2.5	2.5 + 2.5	3.0 + 3.0	3.5 + 3.5	4.0 + 4.0	4.5 + 4.5	4.5 + 4.5	5.0 + 5.0
H_2SO_4 **(in µl)**	1.0	2.0	3.0	4.0	5.0	6.0	7.0	7.0	8.0	9.0	9.5	10.0

STORAGE STABILITY OF REAGENTS AFTER OPENING OR PREPARATION :

a) Wells :
Stored for 8 weeks at 2-8^{o}C in resealable pack

b) MB Substrate :
Stored for 8 hours at 15-30^{o}C in the dark

c) Wash Buffer :
Stored for 2 weeks at 2-8^{o}C.

Equipment and Materials required but not provided

1.Equipment

Incubator, 37º C

Microelisa reader, single wavelength capacity for 405nm

Microelisa washer, capable of dispensing and aspirating a minimum of 300 µl per well

12 channel variable volume (50 to 300 µl) pipette system and tips

Disposable tip micropipette, 3 µl or 5µl, 500 µl and tips

Graduated cylinder, 50 ml or equivalent

Timer

2.Materials

Absorbent paper

Purified water, or equivalent

Strip holder with uncoated strip wells

Disposable reagent reservoirs

Disposable gloves

Sodium hypochlorite solution (5%) or liquid bleach

Biohazard waste containers for materials potentially contaminated with infectious agents

Standard Preparation

None

Calibration Procedure

None

TEST PROCEDURE :

Step 1 Fit required number of strips.

Step 2 Pipette 100µl of Sample Diluent into all well.

Step 3 Pipette 50µl of sample / control into the assigned wells.

Step 4 Agitate gently for about 30 seconds. Incubate for 60±5mts. In humid condition.

Step 5 At the end of incubation, Wash the strips.

[WASH PROCEDURE: Use Dynex Elisa washer Program Number : 16. Alternatively in Stat fax 2600 Elisa washer program no 14.] Machines are programmed to wash 6 cycles with 30 seconds intervals between each wash.

Step 6 Pipette 100µl of TMB into each well.

Step 7 Incubate at 15 to 30^{o}C for 30±2mts.

Step 8 At the end of incubation Stop by adding 100µl of 1mol/l H_2SO_4.

Step 9 Within 15 minutes, read the plate at 450±5 nm (single wavelength) or at 450±5 nm ; 620 to 700 nm as reference filter (dual filter).

Reading procedure: Use Multiskan Ascent Elisa plate reader or alternatively in Stat fax 2100 Elisa Reader in absorbance mode.

Qualification of Negative Controls (NC) Values.

Absorbance of NC must be Lesser than or equal to 0.250. Eliminate outliers and calculate the NC mean (NCx). Absorbance of NC must be lesser than or equal to 1.4 multiplied by NCx. Eliminate outliers and recalculate mean. Absorbance of NC must be greater than or equal to 0.6 multiplied by NCx. Eliminate outliers and recalculate mean If two or more values are outside range, the run is invalid and should be repeated.

Qualification of Positive Control (PC) Value.

Absorbance of PC1 & PC2 subtracted by mean of negative controls should be greater than or equal to 0.600. If PC1 & PC2 is outside this limit, the run is invalid and should be repeated. For PC3 it is 0.4.

QUALITY CONTROL VALIDATION CHART

FIRST LEVEL

NC ODs	CONDITIONS	3.VALID NC ODs	MEAN OF VALID NCs
NC1……. NC2……. NC3…….	All Negative Control must be < 0.250	…….. …….. …….	

SECOND LEVEL

CONDITION	VALID NC ODs	MEAN OF VALID NCs
ALL NC must be ≤ 1.4 X NCx* (**1.4 X NCx* =……..**)	…….. …….. …….	

THIRD LEVEL (final level)

CONDITION	VALID NC ODs	MEAN OF VALID NCs
ALL NC must be ≥ 0.6 NCx* (**0.6 X NCx* =……..**)	……. ……. ……	

NCx =

TEST VALIDITY

CONDITIONS		STATUS
More than half the number of Negative controls should pass		
PC1 – NCX* ≥ 0.600	……..... - ..……...=	**1.PASSED/FAILED**
PC2 – NCX* ≥ 0.600 (If used)	……..... - ..……...=	**PASSED/FAILED**
PC3 – NCX* ≥ 0.400 (If used)	……..... - ..……...=	**PASSED/FAILED**

Note: If assays which match the above four criteria, calculate the Cut off value or repeat the test.

Calculation :

calculate the cutoff OD by using the following formula.

Interpretation:

1. POSITIVE :

If the patient OD is greater than cutoff OD it is declared as positive*.

2. NEGATIVE :

If the patient OD ism less than cutoff OD, it is considered as negative.

*If some Samples shows positive results with very low Optical Densities the had to be repeated in a separate run in duplicate wells. And the interpretations for these samples are as follows

i) Positive in both the wells : Positive

ii) Negative in both the wells : Negative

iii) Positive in one well : Indeterminate

a. Patients may be asked to come after 3 months
b. Or they can be recommended to go for Western blot diagnosis as recommended by the clinician.

Performance Characteristics of Test for Serum and Plasma

Sensitivity

Assay sensitivity was calculated based on testing of samples from individuals clinically diagnosed with AIDS. Samples from 159 individuals were tested and showed, based on an assumed 100% prevalence of HIV-1 antibody in AIDS participants, that the sensitivity of the Vironostika HIV-1 Microelisa System on AIDS samples is 100%. An additional 43 samples from ARC participants and 54 samples from asymptomatic participants were also tested, yielding a 100% sensitivity rate for confirmed positive samples.

Specificity

The specificity of Vironostika HIV-1 Microelisa System was calculated from testing of samples from 6039 normal U.S. donors. Specificity was based on an assumed zero prevalence of antibody to HIV-1 in random U.S. donors. Of the 6,039 donations tested, three were reactive on repeat testing by the Vironostika HIV-1 Microelisa and were confirmed positive by Western blot. Therefore, in this Study, the Vironostika HIV-1 Microelisa System has an estimated specificity of 100% (95% confidence interval of 99.4% to 100%). See Table II in package insert for summary of testing.

REFERENCE : VIRONOSTIKA HIV Uni-Form II Ag/Ab kit insert.

HPTN JHU HIV Genetic System HIV-1 Western Blot Kit SOP

PRINCIPLE:

Name and Intended Use

The Genetic System™ HIV-1 Western Blot Kit is an in vitro qualitative assay for the detection and identification of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in human serum, plasma, or dried blood spots. It is intended for use with persons of unknown risk as an additional, more specific, test on human serum, plasma or dried blood spot specimens found to be repeatedly reactive using a screening procedure, such as Enzyme Linked Immunosorbent Assay (ELISA).

Summary and Explanation of the Test

The major etiologic agent of Acquired Immunodeficiency Syndrome (AIDS) is a retrovirus called Human Immunodeficiency Virus Type 1 (HIV-1). Enzyme immunoassays (EIA) to detect the presence of viral-specific antibodies to HIV-1 have been described by several investigators. Patients with AIDS and AIDS-related conditions exhibit a high prevalence of antibodies to HIV-1, and antibodies to HIV-1 have also been reported in virus positive, asymptomatic individuals.

The original purpose of HIV-1 screening assays was to detect potentially infectious units of blood and to prevent these units from being used in transfusion or in the manufacture of blood products from transfusion. However, these highly sensitive tests have a relatively low positive predictive value for populations with a low prevalence of HIV-1 infection. Some specimens may contain antibodies for HLA Class II histocompatibility antigens found on certain cell lines used to produce the virus for commercial applications, or specimens may react with bacterial contaminants associated with production of recombinant proteins. Other individuals who have had no known exposure to HIV-1 react with HIV-1 core proteins on the EIA for unknown reasons. Since the psychosocial and medical implications of a positive antibody test may be devastating, it is prudent to perform additional testing on such samples to further demonstrate the presence of antibodies specific to HIV-1.

The Western Blot as described by Tsang, et al is useful for elucidating the specificity of the antibody response to HIV-1. In the Western Blot assay, disrupted proteins of HIV-1 are fractionated by electrophoresis according to molecular weight using a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). The resolved protein bands are transblotted to a nitrocellulose sheet. The nitrocellulose sheet is then cut into strips, which are reacted with serum, plasma, or dried blood spot specimens.

If virus specific antibodies are present, they bind to their corresponding viral protein bands. The bands are visualized by using a phosphatase-labeled goat anti-human immunoglobulin conjugate, followed by a substrate for the enzyme. The presence of specific HIV-1 immunoglobulins in serum specimens is indicated by labeling of HIV-1 specific proteins on the strip. Recognized HIV-1 viral antigens produce bands at gp160, gp120, p65, p55, p51, gp41, p40, p31, p24 and p18.

A sample that is reactive in the EIA and positive on the Western Blot is presumed to be positive for antibodies to HIV-1. Individuals with positive tests should be referred for medical evaluations. A diagnosis of AIDS can be made only if an individual meets the case definition of AIDS established by the Centers for Disease Control.

Chemical and Biological Principles of the Procedure

Purified, inactive HIV-1 strain LAV grown in the CEM cell line is disrupted and electrophoretically resolved into bands. The proteins are transblotted onto nitrocellulose sheets, which are cut into strips. Samples are diluted in Specimen Diluent/Wash and applied to the nitrocellulose strip. If specific HIV-1 antibody is present, it binds to proteins resolved on the strip. Unbound sample is removed by washing. The phophatase labeled conjugate is then added to the strip and allowed to incubate. The conjugate attaches to antibody already bound to viral proteins on the strip. Excess conjugate is removed by washing. Color Development Reagent is then added to the strip. Reaction sites, where enzyme labeled antibody is bound, are identified by purple bands. The position and intensity of the bands are compared to reference strips developed using Positive Control sera.

SPECIMEN:
Serum or Plasma

Type:

No special preparation of the patient is necessary. Serum or plasma may be used. The following anticoagulants have all been evaluated and found to be acceptable: EDTA, heparin, sodium citrate, CPDA-1, and ACD. Samples which are collected into anticoagulant tubes should be filled as labeling indicates to avoid improper dilution. Remove the serum or plasma from the clot or red cells as soon as possible to avoid hemolysis. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. No clinically significant effect has been detected in assay results with increased levels of protein, lipids, bilirubin, or microbiological contaminants, or after heat inactivation of patient samples.

Handling Conditions:

Serum or plasma may be stored at 2-8°C for up to thirty days per internal validation. For long-term storage, the specimens should be frozen (at -20°C or colder). Samples should not be used if they have incurred more than 5 freeze/thaw cycles. Specimens repeatedly frozen or thawed or those containing particulate matter may give erroneous results. Mix samples thoroughly after thawing. Note: on hold samples are spun and stored at 2-8°C for thirty days since they are kept in their original containers.

If specimens are to be shipped, they should be packed in compliance with Federal Regulations covering the transportation of etiologic agents. Studies have demonstrated that specimens may be shipped refrigerated (2-8°C) or at ambient temperatures (<25°C) for up to 7 days. For shipments that are in transit for more than 7 days, specimens should be kept frozen (-20°C or lower). Refrigerate samples at 2-8°C at receipt, or freeze for longer storage.

In addition to detecting antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in human serum or plasma, the Genetics Systems™ HIV-1 Western Blot may be used to test whole blood specimens collected onto filter paper and dried. Drops of whole blood should be obtained by using a licensed collection kit or collected according t the National Committee for Clinical Laboratory Standards, by either finger puncture or heel stick.

(1) Label a separate piece of filter paper for each specimen with the appropriate specimen identification. Use a ballpoint pen or other water indelible marker. Handle the filter paper by the edges; do not touch the areas that will be used to collect the specimens.

(2) Prepare the area (either finger or heel) for puncture. The puncture must be performed with sufficient force and penetration to sustain a flow of at least several drops of blood. Allow a large drop of free flowing blood to collect the drop, and allow another large drop to form at the puncture site. Continue to collect drops in this manner until the wound ceases to bleed or until collection is sufficient.

(3) Collect each drop of blood in a separate area of the filter paper (if the paper is marked with several circles, place each drop in a different circle). Do not layer successive drops of blood in the same spot. In addition to the sample that is required to perform the EIA (i.e. one spot of blood ≥1/4 of an inch in diameter) at least one spot of blood that is ≥1/4 of an inch in diameter must be obtained in order to perform the Genetic Systems™ HIV-1 Western Blot.

(4) If the wound stops flowing before sufficient blood has been obtained, a second puncture should be performed. The wound may be massaged very gently to encourage formation of large blood droplets. Do not squeeze the wound to obtain more blood as this may result in hemolysis of the specimen or a mixture of other body fluids with the specimen.

(5) After the blood has been absorbed into the filter paper, it should be dried at room temperature for a t least three hours. The filter paper may be allowed to dry at room temperature overnight. When dry, the spots will be uniform dark brown. No areas of red coloration should be seen; the appearance of the spots should be similar to that of a dried blood stain.

(6) When the blood spots are completely dry, a sample may be punched and eluted as described in Specimen Handling and Preparation. If dried blood specimens are to be shipped, they should be enclosed and sealed in either a moisture barrier container, such as a heavy duty zip lock bag with dessicant or a high quality envelope.

Handling Conditions

Studies performed at Genetics Systems indicate that completely dried specimens may be stored frozen (-20°C) or refrigerated (2-8°C) for at least two months under low humidity conditions. Storage under accelerated temperature conditions (room temperature or higher) leads to increasing non-specific reactivity over time. Therefore, it is recommended that dried blood spots be stored for no more than one week at room temperature or up to two months refrigerated or frozen; routine storage at room temperature or higher is not

recommended. If specimens are stored at any conditions other than the ones listed above, the user must validate the stability of the specimens under those storage conditions. If specimens are to be stored in a humid environment (≥60% relative humidity), the user should include a dessicant.

This kit is not licensed for use with specimens other than serum, plasma, and dried blood spots. This kit is not intended for use on saliva/oral fluids or urine samples.

EQUIPMENT AND MATERIALS:

Materials Required but not Provided

(1.) Precision pipettes to deliver 5 µl, 10µl, 100 µl, 1 ml, 5 ml, and 10 ml) or automated pipettor-dilutor, and pipette tips.
(2.) Appropriate containers to prepare diluted specimens (12 x 75mm tubes) and reagents (polypropylene).
(3.) Water bath capable of maintaining 37 ± 1°C.
(4.) Refrigerator 2 - 8°C
(5.) Graduated Cylinders
(6.) Glass or plastic beaker, 15 to 600ml
(7.) Dispatch™,.Alternative disinfectants include: Household bleach (5% to 8% sodium hypochlorite) which may be diluted to a minimum concentration of 10% bleach (or 0.5% sodium hypochlorite),70% ethanol or Cidecon™ (1:128)
(8.) Balance
(9.) Laboratory timer
(10.) Magnetic stir plate and stir bar
(11.) Deionized or distilled water. Clinical laboratory reagent water Type I or Type II is acceptable. .
(12.) Gloves, nitrile.
(13.) Scissors
(14.) Graduated pipettes to deliver volumes up to 25ml
(15.) Forceps
(16.) Transfer pipettes
(17.) Rotary platform, capable of rotating at 50 to 60 rpm
(18.) Aspiration System
(19.) Repeating pipette to deliver 2ml volumes
(20.) Vortex
(21.) Alternatively, Western Blot instrument such as AutoBlot 2000 by MedTec Biolab equipment
(22.) Isopropyl alcohol

Materials Provided:

HIV-1 Western Blot Kit Genetics Systems™ Western Blot Product

R1 HIV-1 Western Blot Strips (40), 2 packages of 20 strips

Package contains 20 strips; sufficient for 20 tests. Nitrocellulose strips preblotted with resolved HIV-1 viral proteins. Blotting paper buffer contains 0.1% sodium azide and 0.1% Proclin 300. Ready to use as supplied.

C0 Western Blot Negative Control, 1 vial (0.2ml)
Normal human serum or plasma. Non-reactive for HbsAg and antibodies to HIV, HCV, and HTLV-I/II. Contains 0.1% sodium azide and 0.5% Proclin 300. Dilute in working specimen diluent/wash.

C1 HIV-1 Western Blot Low Positive Control, 1 vial (0.2ml)
Heat inactivated human serum or plasma containing antibodies reactive to HIV-1. Non – reactive for antibody for HbsAg and non-reactive for antibody to HCV and HTLV I/II. Contains 0.1% sodium azide and 0.5% Proclin 300. Dilute in working specimen diluent/wash.

C2 HIV-1 Western Blot High Positive Control, 1 vial (0.2ml)
Heat inactivated human serum or plasma containing antibodies reactive to HIV-1. Non – reactive for antibody for HbsAg and non-reactive for antibody to HCV and HTLV I/II. Contains 0.1% sodium azide and 0.5% Proclin 300. Dilute in working specimen diluent/wash

R2 Western Blot Specimen Diluent/Wash (5X) 2 bottles (100ml)
TRIS buffered saline and mil proteins. Contains 0.5% Proclin 300. Dilute in deionized water.

R3 HIV-1 Western Blot Conjugate 1 bottle 80ml
Anti-human IgG, IgA, and IgM (goat) alkaline phosphatase conjugated solution. Contains 0.1% sodium azide and 0.5% Proclin 300. Ready to use as supplied.

R4 HIV Western Blot Color Development Reagent 1 bottle (100ml)
5-bromo-4-chloro-3-indolyl phosphatase (BCIP), Nitro blue tetrazolium (NBT), and organic base/TRIS buffer. Ready to use as supplied.

Disposable reaction trays, 8 trays
Disposable slotted reaction trays and lids. Ready to use as supplied

STORAGE CONDITIONS

Store the kit at 2 - 8°C upon arrival. All components should be stored at 2 - 8°C and returned to 2 – 8°C after use. Bring 5x Specimen Diluent/Wash to room temperature (15 - 30°C) before use. Return unused strips to package and reseal.

DO NOT MIX KIT LOT NUMBERS.

Preparation:

HIV-1 Western Blot Strips

HIV-1 Western Blot Strips are packed in a resealable plastic pouch between buffer-soaked blotting paper. When ready to use, cut the bag below the seal line and retain the upper portion of the bag. Cut the vertical sides of the lower portion of the bag so that it may open like a book. This prevents the technologist from having to handle the blotting paper directly with the hands.. Separate the strips to be assayed and place them into troughs of a reaction tray. Place the "book" of remaining strips (still inside the blotting paper) in the upper portion of the bag. Seal the bag using the zip closure. Keep tightly sealed. DO NOT LET STRIPS DRY OUT. Store at 2-8°C. NOTE: avoid contaminating the strips or moist blotting paper during the handling. This may cause false reactivity.

Working Specimen Diluent/Wash

Use Working Specimen Diluent/Wash for wash solution and dilution of serum and plasma samples or elution of dried blood spots. Prepare Working Specimen Diluent/Wash as follows: bring 5X Specimen Diluent/Wash to room temperature, invert to mix before using, and then dilute 1:5 with deionized or distilled water. (i.e. 1 part 5X Specimen Diluent/wash plus 4 parts deionized or distilled water). Clinical laboratory reagent water Type I or Type II is acceptable. For each strip to be tested, prepare 15ml (i.e. 3ml 5X Specimen Diluent/Wash plus 12ml of deionized water). Prepare the volume of diluted reagent that will be adequate for the entire run. Mix working solution when combined and again just prior to use. The working Specimen Diluent/Wash may be stored at 2 – 8°C for up to two weeks. Bring to room temperature and mix thoroughly prior to use.

Preparation of Working Specimen Diluent/Wash

Number of strips to be used	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Amount of 5x Spec Dil/Wash ml	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
Deionized Water ml	48	60	72	84	96	108	120	132	144	156	168	180	192	204	216	228	240
Total Volume ml	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300

Warnings for Users

Warning: FDA has licensed this test for use with serum, plasma, and dried blood spots specimens only. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.

1. The HIV-1 High and Low Positive Controls are heat treated to inactivate viruses. However, handle all the reagents as though capable of transmitting infection. All tests should be conducted using the precautions recommended for blood borne pathogens, as defined by OSHA regulations.
2. Do not pipette by mouth.
3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

4. Wear protective clothing and disposable gloves while handling the kit reagents. Wash hands thoroughly after performing the test.
5. BIOLOGICAL SPILLS: Spills should be wiped thoroughly with Dispatch™. Household bleach (5% to 8% sodium hypochlorite) which may be diluted to a minimum concentration of 10% bleach (or 0.5% sodium hypochlorite). Alternative disinfectants include: 70% ethanol or Cidecon™ (1:128) NOTE: Do not place solutions containing bleach in the autoclave (this includes Dispatch™).
6. Dispose of all specimens and materials used to perform the test as though they contain an infectious agent. The preferred method of disposal is autoclaving for one hour at 121.5°C. Neutralized waste and liquid waste not containing acid may be mixed with an appropriate chemical disinfectant. The final mixture must contain more than the recommended minimum concentration of disinfectant. Allow 30 minutes for effective decontamination.
7. Sodium azide is included as a preservative in the HIV-1 Western Blot Strips, the kit controls and the HIV-1 Western Blot Conjugate. Sodium azide has been reported to form lead or copper azides in laboratory plumbing. These azides are explosive. To prevent build up, flush plumbing with a large volume of water if solutions containing azides are disposed of in the sink after inactivation.

QUALITY CONTROL

Both the Negative, Low and High positive controls must be assayed regardless of the number of samples tested. The High Positive Control will be compared to the test strips run to determine band identification and placement.

The following conditions must be met for assay results to be considered valid:

Kit Controls

Negative Control (- or =): No bands are observed on the strip

Low Positive Control(+): a band must be present at gp120and the interpretation of the Low Positive control must be positive. Other bands may or may not be present.

High Positive Control(++): Bands must be present at gp160, gp120, gp41, and p24. Other bands may or may not be present.

A negative patient sample may also be assayed and contain no HIV related bands.

Any run that does not meet this criteria needs to be repeated and documented in the technical delays section of the Quality Assurance/Assessment manual.

TROUBLESHOOTING:

PROCEDURE:

Preliminary Statements

1. The expected run time for this procedure is approximately 3 hours. Each run of this assay must proceed to completion without interruption after it has been started.

2. Positive and Negative controls must be evaluated with each run. Compare the intensity of patient samples to controls for each set of strips to determine patient results. **The negative control must be placed between the High positive control and the low positive control.**
3. The minimum controls to be included in each run of this assay are: one HIV-1 Low Positive control, one High Positive control, and one Negative control. If multiple packages of strips are used in one run, controls must be included for each package of strips.
4. Do not splash controls, specimens, or reagents between troughs of the reaction tray.
5. Cover reaction trays for each incubation step using the lid provided, or equivalent.
6. Ensure that each one of the strips is entirely covered with liquid during the incubation steps
7. Adhere to the recommended time constraints for the incubation steps.

Specimen Preparation

Caution: Handle all specimens, controls, and HIV-1 Western Blot Strips as though capable of transmitting infectious disease.

Dried Blood Spot Specimen Preparation

Use scissors to remove a ¼" disk of each whole blood specimen to be tested. Cut the disk from a uniform area of one of the completely dried spots of blood. Place each ¼" disk in a separate, clean, empty trough of a reaction tray.

1. Use a precision pipette or repeating dispenser to add 500ul of Working Specimen Diluent/Wash to each trough containing a filter paper disk. Mix the specimens well to wet the filter paper thoroughly (for example, use a rotator). Cover the reaction tray with a lid to minimize evaporation. Tilt tray approximately 5/8" (15mm) to concentrate liquid in area of spot. Elute the specimens for 2 hours at room temperature (15 – 30°C) with rotation at 50 – 60 rpm, or overnight (static) at 2 - 8°C.
2. At the end of the two-hour elution or when the refrigerated eluates warm to room temperature, the filter paper disk should be almost white (a faint brown color may remain in the disk). Specimens eluted in this manner are ready to use without further dilution in the Genetics Systems™ HIV-1 Western Blot. The filter paper disk may be removed from the trough with clean forceps before starting the assay or the filter paper disk may be left in the trough and removed just prior to the addition of Conjugate (before Step 7 of the Procedure). Clean the forceps with deionized water, Dispatch, alcohol and again with deionized water, then wipe dry in between removal of each filter paper disk in order to avoid cross-contamination.

    1. Prepare Working Specimen Diluent/Wash and allow it to come to room temperature before use.
    2. Label the reaction trays appropriately to maintain control and sample identification.
    3. Add diluted specimens to reaction trays by:

Serum and plasma: using a clean disposable pipette, carefully add 1ml of Working Specimen Diluent/Wash into each trough. Using a clean disposable precision micropipette, transfer 10ul of each control or specimen to the respective trough containing 1ml of Working Specimen Diluent/Wash (final dilution 1:101).
Dried blood spots: see dried blood spot specimen preparation above. Note the required volume of dried blood spot eluate is 0.5ml strip**. Caution: Adding Western Blot Strips to diluted specimen may increase the risk of cross contamination. While this method is acceptable, it would be better to place the strip(s) in a clean trough then add the diluted specimen.**

4. Place one strip, with the indicator line facing up, into each trough containing a diluted serum or plasma specimen, dried blood spot eluate, or control. Note: Gently detach each strip with forceps at the top of the strip (near the number). Transfer the strips carefully; avoid contact with contaminated surfaces. To avoid cross-contamination, do not touch the forceps to the liquid in the trough.
5. Cover the reaction tray and incubate with gentle agitation on a rotator at 50-60 rpm for 60-65 minutes at room temperature. Note: Be certain that each strip is immersed in the liquid and is moving freely during rotation. Liquid should not contact the tray during rotation.
6. Aspirate the liquid from the troughs. Wash aspiration manifold by aspirating water 3 – 5 seconds between trays of strips. Immediately fill each trough with at least 1ml Working Specimen Diluent/Wash.
7. Rock the reaction tray back and forth approximately 8 times. Be certain that each strip is immersed in liquid and is moving freely when rocked.
8. Aspirate the liquid completely. Repeat wash step 4 times. NOTE: Incomplete or ineffective washing will compromise the assay. Do not allow the strips to dry out.
9. After the final wash, add 1.0 Working Specimen Diluent/Wash to each trough. Place the reaction tray on the rotator for 4 to 6 minutes. Note: If filter paper disks have not been removed, remove them now with forceps. Clean the forceps with deionized water and wipe dry between removal of each filter paper disk in order to avoid cross contamination.
10. Following the soak, aspirate the contents of each trough completely. Immediately add 1.0 ml of Conjugate to each trough.
11. Cover the reaction tray with the lid and incubate with gentle agitation on the rotator at 50 – 60 rpm for 45 – 50 minutes at room temperature. Note: Be certain that each strip is immersed in the liquid and is moving freely during rotation. Liquid should not contact the tray lid during rotation.
12. After incubation aspirate the liquid from each trough. Wash the strips as indicated in steps 6 through 8.
13. After the final wash, add 1.0 ml of deionized water to each trough. Place the reaction tray on a rotator for 4-6 minutes.
14. Following the soak, aspirate the deionized water from each trough completely. Immediately add 1.0ml of Color Development reagent to each trough. Gently rock the reaction tray back and forth at least twice. Be sure that each strip is

immersed in Color Development reagent. Allow the color to develop for 3 –4 minutes. Do not over-incubate Color Development Reagent.

15. Stop the reaction by aspirating the Color Development reagent and flooding each trough with a minimum of 1.0 ml of deionized water. Allow to stand for 5 – 10 minutes. Aspirate the water.

16. Allow the strips to air dry in the reaction tray before reading the bands. Handle the strips carefully. Use forceps to remove from the troughs.

17. Store complete strips away from direct sunlight to avoid fading. For permanent storage, it may be desirable to mount the strip on durable paper and cover them with a plastic sheet for protection.

PROCEDURE – STEPWISE WITH INSTRUMENTATION:

1. Prepare Working Specimen Diluent Wash, dH_2O, conjugate, and substrate by warming to room temperature for approximately 30 minutes.
2. Prepare an assay worksheet, ensuring that the patient sample and control identification is linked to the number embossed on the test strip.
3. Prepare instrument by gently mixing and placing all reagents on the instrument.
4. Power on the instrument.
5. Panel will read "ready for a new test" press yes
6. ASSAY NAME 01:GSC-1
7. Press 'enter', Panel will read "Pump pads in place", Press 'yes".
8. Prime pumps press Yes.
    a. Prime wash line – press Yes. Press Yes 2 times until liquid is dispensed.
    b. Prime wash line – press 'NO'.
    c. Prime dH_2O press Yes. Press Yes 2 times until liquid is dispensed.
    d. Prime dH2O press 'NO'.
    e. Prime substrate press Yes Press Yes 1 times until liquid is dispensed
    f. Prime substrate press'NO'.
    g. Prime conjugate press Yes. Press Yes 2 time until liquid is dispensed
    h. Prime conjugate press 'NO'.
    i. Prime pumps press No.
9. Strip count – adjust to appropriate number of strips being run, enter using the arrow keys
10. Load tray – press enter. Do not add strips yet.
11. Start assay press Yes. Instrument will add 1.0 of Working Specimen Diluent Wash.
12. Add strips, Press enter.
13. Unit rocks for 1 minute. An alarm will sound in 30 seconds.
14. Sample prepared, press Yes and rocker will stop.
15. At sample installed add samples. Add 10ul of sample directly to tray not over the strip.
16. Once all samples have been installed enter Yes.
17. The unit rocks and the samples incubate for 60 minutes at room temperature..
18. Instrument will proceed with the washing of strips 4X. At QWASH it will dispense 1.0 mL of Working Specimen Diluent/Wash. This will take approximately 15 mins.
19. At WASH, there is a 5 minute incubation with the samples and Working Specimen Diluent /Wash, rocking.

20. 1.0 ml of conjugate will be dispensed to each trough.
21. Incubate for 45 minutes.
22. Instrument will proceed and wash strips, 4 times with 1.0 mL of Working Specimen Diluent/Wash. This will take approximately 15 minutes.
23. At WASH there is a 5 minute incubation with samples and dH2O.
24. The instrument will dispense 1.0 ml of chromagen to each trough. The unit rocks during this step for 3 minutes
25. Once the incubation is complete, the strips will be washed with deionized water rocking for 1 minute.
26. The strips will be washed again with dH2O rocking for 7 minutes.
27. The system will alarm. Remove tray from instrument.
28. Remove strips by number area only and place on a paper towel to dry. Drying will take 1 to 2 minutes.
29. Follow the following shutdown procedure for the instrument: Purge tubing press 'Yes'.
    a. Prepare 70% Ethanol and dH2O.
    b. Auto flush lines: press 'Y'.
    c. Place lines in 70% ethanol
    d. Soak for 5 minutes
    e. Place lines in dH_2O
    f. Soak for 5 minutes
    g. Lines will flush automatically. Release pump pads. Press enter.
    h. Shut off instrument.
30. Document appropriate maintenance on the Preventive Maintenance and Quality Control Checklist sheet on the clipboard in the HIV Ab room, Pathology 311A.

REPORTING RESULTS

Interpretation of results

Evaluate each strip for the presence of bands. A band is defined as a distinct purple line that extends horizontally across the strip where human antibodies have bound to resolved proteins. The lines may be of varying thickness. Smudges, spots and lines, which do not extend the width of the strip, should not be interpreted as bands. Sometimes bands may appear on the backside of the strip. This can be disturbing when looking at the negative control or patients. These should be ignored as well. Any lines that are higher than gp160 or lower than p18 on the strip are outside of the reading area of the strip and therefore are not to be interpreted as bands. These are reported as negative.

Use the HIV-1 High and Low positive controls to identify bands that may be present on patient specimen strips. Score relative intensity of bands present on patient specimen strips by comparing to bands on control strips as follows:
Intensity of band:

Absent - or =

Less than the intensity of gp120 on the HIV-1 Low Positive Strip +/-

At least as intensely reactive as gp120 on the HIV-1 Low Positive Control Strip but less intense than gp120 on the HIV-1 High Positive Control Strip +

Greater than or equal to the intensity of gp120 on the High Positive Control strip ++

Interpret the immunoblot as Negative, Indeterminate, or Positive based on the pattern that is present.

POSITIVE At least two of the major bands: gp160/gp120, gp41, or p24 must be present. Bands must be at least as intense as the Low positive control gp120 band to be considered positive. The band at gp41 must be broad and diffuse.

Indeterminate One or more bands are present but the blot does not meet the criteria for a positive result as described above.

Negative No bands are present

NOTE: Negative dried blood spot specimens frequently exhibit a weakly reactive (+/-) fine band migrating within the wide gp41 region. This reactivity is clearly distinguishable for gp41, which is a broad diffuse band. Dried blood spot specimens that are reactive only with this discrete p42 band or contain only lines or marks that are higher than gp160 or lower than p18 may be interpreted as negative.

Indeterminate results should not be considered either positive or negative. Additional immunoblot testing and clinical evaluation must be utilized to correctly evaluate an indeterminate result.

ANATOMY of HIV-1

Virus Gene	Gene product	Gene description
Env	gp160 gp120 gp41	Env protein precursor Outer env protein Transmembrane protein
Pol	p65 p51 p31	Reverse transcriptase Reverse transcriptase Endonuclease
Gag	p55 p24 p18	Core protein precursor Core Core

Reference Ranges:

There should be no viral band reactivity**.**

PROCEDURE NOTES:

Precautions for users

(1) Do not use the kit beyond the stated expiration date.
(2) Bring Specimen Diluent/Wash to room temperature before use.
(3) The only reagents that may be used with different lots of the HIV-1 Western Blot are the Specimen Diluent/Wash and Color development Reagent. Do not mix ay other reagents from different lots.
(4) Exercise care in opening and removing aliquots from vials to avoid microbial contamination.
(5) Prior to removing a strip from the pouch, clean the work surface and forceps with 70% ethanol or isopropyl alcohol.
(6) Using forceps, remove each strip from the left side of the series in the package first, and maintain the same order in the reaction tray.
(7) For the pipetting of controls and specimens, use individual pipette tips to eliminate carryover of samples.
(8) Before measuring samples or reagents, pre-wet the pipette tips with the sample or reagent.
(9) Avoid cross-contamination during sample incubation and aspiration:
    a. Be particularly careful when transferring reaction trays to the rotator during sample incubation to avoid spilling fluid from the troughs.
    b. Liquid in the troughs should not contact the tray lid during rotation.
    c. A strip may be placed in every second trough to further reduce the risk of cross-contamination
    d. Use clean tips to aspirate samples to avoid cross-contamination.
(10) Do not reuse trays or lids.
(11) It is important that the pipette used to dispense Working Specimen Diluent/Wash be scrupulously clean. A disposable pipette is preferable.
(12) Use a clean, disposable container (e.g. reagent reservoir) and pipette for dispensing Conjugate. Exposure of Conjugate to serum will inactivate Conjugate.
(13) It is imperative that the final wash, prior to the addition of Color Development reagent, be performed with deionized or distilled water. Residual Specimen Diluent/Wash in the troughs may decrease band intensity by inhibiting the substrate reaction.
(14) Handle negative and positive controls in the same manner as patient specimens.
(15) If a specimen is inadvertently not added to a strip, the assay result will read negative.
(16) Inadequate adherence to package insert instructions may result in erroneous results.

Technical Tips

(1) Do not expose assay to bleach or bleach fumes.
(2) It is imperative that the container used to prepare the Sample buffer be scrupulously clean.
(3) The dispenser for the sample buffer should be disposable or scrupulously clean.
(4) The sample buffer should be used within 24 hours after preparation.

(5) When running the overnight assay option, the sample buffer must be refrigerated overnight then brought to room temperature the following morning prior to continuation of the assay.
(6) Pipette accuracy should be verified before use.
(7) Change gloves prior to strip handling. Forceps and scissors should be cleaned with isopropyl alcohol prior to use.
(8) Prepare an assay worksheet, ensuring that patient sample and control identification is linked to the number embossed on the strip.
(9) The conjugate should be prepared in a clean container (preferably a disposable 50ml conical tube) just prior to use.
(10) The wash procedure is critical. Fluid in troughs must be aspirated completely. The strips should be fully immersed in the liquid and moving freely.
(11) Western Blot strips are easily cross contaminated. This test is extremely sensitive to small amounts of antibody. This is why it is important to observe precautions to prevent the inadvertent transfer of antibody from one sample to another.
(12) Aspirate conjugate completely. DO not over incubate the conjugate.
(13) Clean and disinfect the aspirator device at least once a week.

Technical Tips for Preserving Western Blot Strips

Assayed Western Blot strips can fade when exposed to light. To minimize fading, handle the strips in a consistent and light protected manner. The following will help reduce the expected Western Blot phenomenon of fading:

(1) Cover the strips when not being viewed. During the drying step, carefully cover the tray with a paper towel.
(2) Mount the strips as soon as they are dry and place in a dark place.
(3) Always try to follow the same schedule when mounting and reading the Western Blot strips. It is not good practice to read some strips. One hour after assaying and then read a different set of strips three days after assaying. There is a possibility for some difference due to the timing.
(4) Photocopying can hasten the fading process.
(5) Reactive vapors may cause strips to fade. Store strips away from bleach and or chemical fumes.
(6) Expect some fading with time. For historical data, rely on recorded interpretations for accurate results.

Technical Tips for Preventing Cross Contamination

(1) Clean work area with isopropyl alcohol prior to start of assay. Work on a clean sheet of toweling.
(2) If sample dilutions are prepared in the western blot testing area, clean the work area again prior to handling the strips.
(3) Put on a fresh pair of gloves prior to opening the package and handling strips.
(4) Cut plastic bag under seal line and on two edges so that when you hold packet in hand to separate strips, the blotting paper is encased in plastic. Clean forceps with isopropyl alcohol prior to transferring strips.
(5) Mix and transfer entire volume of sample dilution carefully to the appropriate trough and strip using a transfer pipette.

(6) If it is necessary to walk with the trays, hold them so that the troughs run perpendicular to the direction of your body's movement to avoid "sloshing".
(7) Do not stack trays on the rotator.
(8) Be certain that the rotator speed is high enough to cause complete movement of the liquid in the troughs (50-60rpm), yet not so high as to cause the liquid to touch tray lids.

Troubleshooting Guide

Increased background color

(1) Inadequate or incomplete washing (especially following conjugate incubation)
(2) A room temperature above 25°C.
(3) Conjugate incubation exceeds 45 minutes.
(4) Wash/aspiration system contaminated

Decreased color development of bands

(1) Decreased sample, conjugate or chromogen incubation
(2) Inadequate or incomplete washing following sample incubation
(3) Conjugate not properly prepared
(4) Conjugate left at room temperature for extended period
(5) Chromagen left at room temperature for extended period
(6) Room temperature below 20°C
(7) Strips were allowed to dry
(8) Prepared sample dilutions were tested beyond 1 hour after initial preparation
(9) Presence of reactive vapors (i.e. bleach, aldehydes)
(10) Reagents not properly mixed

Extraneous non-viral banding

(1) Inadequate or incomplete washing
(2) Cross reactivity with other protein bands found in the patient
(3) Specimens that have been repeatedly frozen and thawed
(4) Conjugate incubation exceeds 45 minutes
(5) Wash/aspiration system contaminated

Maintenance

Follow instructions for shut down procedure.

LIMITATIONS OF THE PROCEDURE

NOTE: Only a serum or plasma sample should be used to test blood or plasma intended for transfusion or further manufacture.

(1.) The assay must be performed in strict accordance with these instructions to obtain reproducible results.
(2.) Although a persistently Positive immunoblot for antibodies to HIV-1 indicates infection with the virus, diagnosis of Acquired Immunodeficiency Syndrome or AIDS can only be made on clinical grounds if an individual meets the case definition of AIDS established by the Centers for Disease Control.

(3.) Individuals with Positive immunoblots for antibodies to HIV-1 should be referred for medical evaluation, which may include additional testing. The clinical implications of antibodies to HIV-1 in an asymptomatic individual are not known. However, a large proportion of such individuals have virus detectable in their blood cells and some such individuals will develop immunodeficiency with the passage of time.

(4.) It is generally recognized that detection of HIV antibody in infants born to seropositive mothers is not adequate to diagnose HIV infection in the infants, since maternal IgG frequently persists for as long as 18 months after birth. Supplemental assays designed specifically for neonatal specimens may be helpful in resolving such cases.

(5.) Indeterminate immunoblots should not be used as the sole basis of diagnosis of HIV-1 infection. However, such findings may provide useful information in the context of medical evaluation in which clinical information is available.

(6.) Due to variations in test performance and the uncertainty associated with Indeterminate immunoblots, it is recommended that all Indeterminate immunoblots be repeated using the original specimen. Persons with an indeterminate immunoblot should be retested using a fresh specimen after six months.

(7.) A negative immunoblot does not exclude the possibility of infection with HIV-1. Antibody testing should not be used in lieu of blood donor exclusion and self-exclusion procedures.

(8.) Strips, which are obscured by the development of dark blotches or marks, should not be interpreted.

(9.) A person who has antibodies to HIV-1 is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation and possibly additional testing to decide whether a diagnosis of HIV infection is accurate.

PERFORMANCE CHARACTERISTICS

1.Expected values

The performance of the Genetics Systems™ HIV-1 Western Blot kit was evaluated in clinical studies in low risk, high risk, and AIDS populations. Sera were tested with licensed HIV-1/HIV-2 EIA test kits, FDA licensed HIV-1 Western Blot kits and with this kit. A total of 1,430 sera/plasma samples (115 serum; 315 plasma) and 345 dried blood spots (DBS) were tested by 7 independent clinical sites located in both high and low incidence areas.

Reproducibility

The reproducibility of the Genetic Systems™ HIV-1 Western Blot was evaluated at 7 sites with a panel of 6 specimens tested in duplicate on 5 runs on each of 3 lots. 2 readers at each site scored each band independently. The percent of times each band was scored as present is shown below.

Table 1 Precision
Genetics Systems™ HIV-1 Western Blot

Panel ID	% Frequency of bands present (number ≥ +/-)								
	Gp160	Gp120	P65	P55/51	Gp41	P40	P31	P24	P18
Negative Normal	0	0	0.5	0.5	0	0	0	0	0

Donor									
Negative Pooled Plasma	0	0	1.0	1.0	0.5	0	1.4	2.4	1.0
HIV-2 Positive Sample	1.0	0	95.7	100	45.7	100	99.0	100	4.8
Weak HIV-1 Positive Sample	96.2	1.4	91.9	70.5	2.9	100	77.6	100	2.9
HIV-1 High Positive Sample	100	100	100	100	100	100	100	100	99.5
HIV-1 Low Positive Sample	100	100	100	100	100	100	100	100	93.8

Sensitivity and Specificity

Serum/Plasma

A study in low risk populations included 298 sera/plasma collected prospectively from normal blood donors, as well as 274 EIA repeatedly reactive sera/plasma selected retrospectively from blood center repositories. This latter group of samples was chosen to include sera/plasma testing positive or indeterminate on a licensed HIV-1 Western Blot, as well as unselected with regard to reactivity on a licensed HIV-1 Western Blot test.

The Genetics Systems™ HIV-1 Western Blot was positive for 63/63 (100%) blood center repository samples identified as positive by the licensed HIV-1 Western Blot, demonstrating comparable sensitivity. The Genetics Systems™ HIV-1 Western Blot was negative for 68/78 (87.2%) samples found to be negative by the licensed HIV-1 Western Blot, demonstrating comparable specificity. The Genetics Systems™ HIV-1 Western Blot was indeterminate for the remaining 10 samples).

Of the 298 prospective normal donor samples, all were EIA non-reactive. The Genetics Systems™ HIV-1 Western Blot was negative for 266 (89.3%) samples, indeterminate for 32 (10.7%) samples, and positive for none (0.0%). Twenty-six (26) of the 32 indeterminate samples were additionally tested with a licensed HIV-1 western Blot and found to be either indeterminate or negative

Table 2
Evaluation of the Genetics Systems™ HIV-1 Western Blot in Low Risk Populations
N=572

Clinical Group	Genetics Systems™ HIV-1 Western Blot Interpretation	Licensed Western Blot Interpretation (historical data)		
		Positive	Ind	Neg
Donors EIA Repeatedly Reactive (retrospective study) (n=274)	Positive n=63	63	0	0
	Indeterminate n=83[1]	0	73	10
	Negative n=128	0	60[2]	68
Donors EIA Non-reactive (prospective Study) (n=298)	Positive n=0	0	0	0
	Indeterminate n=32[3]	0[3]	12[3]	
	Negative n=266[4]	14[3]		
		0[4]	4[4]	
		17[4]		

[1]One sample was originally entered into the study as Western Blot positive (historical data) and found to be indeterminate on Genetic System™ HIV-1 Western Blot. It was also indeterminate when retested with the licensed Western Blot suggesting that the sample may have deteriorated during storage.
[2]These were repository specimens that had been stored frozen for various periods of time. Results of Genetic Systems™ HIV-1 Western Blot are compared to historical licensed HIV-1 Western Blot data. No follow up studies were done to determine if the observed differences were due to sample deterioration during storage.
[3]Six of the samples that were indeterminate on the Genetics Systems™ HIV-1 Western Blot were not tested on the licensed Western Blot.
[4]Two hundred forty-five of the 266 samples Negative on the Genetics Systems™ HIV-1 Western Blot were not tested on the licensed Western Blot.

Additional studies in HIV high risk and AIDS populations included 525 samples collected prospectively and retrospectively. Prospectively collected samples included 152 sera from AIDS patients, 20 sera from ARC patients, 26 sera from HIV-1 asymptomatic patients, and 199 repository sera from high risk subjects. These included samples collected in a sequential manner from STD clinic patients (n=49); homosexual males (n=50); and hospital emergency room patients (n=100) in a high prevalence area. Retrospective samples included 128 repository specimens testing EIA repeatedly reactive and positive on a licensed HIV-1 Western Blot. These samples were from various geographic locations including France n=12, Ghana n=5, Nairobi n=7, Nigeria n=12, Australia n=12, Thailand n=12, Zimbabwe n=12, Sierra Leone n=12, Mozambique n=2, Central African Republic n=14, and USA n=28.

These studies demonstrated 172/172 (100%) positive results in AIDS and ARC populations and 176/176 (100%) positive results for high risk samples confirmed positive on a licensed HIV-1 Western Blot. Overall, there was 100% concordance of positive results between the Genetics Systems ™ HIV-1 Western Blot and the licensed Western Blot. Of the 176 EIA non-reactive specimens from high-risk populations, 8 were indeterminate and 137 were negative on both tests, demonstrating 82.4% concordance. There were 31 discordant results, all of which were indeterminate or negative on the two Western Blot kits as shown below

Clinical Group	Genetics Systems™ HIV-1 Western Blot Interpretation	Licensed Western Blot interpretation		
		Positive	Indet.	Negative
AIDS/ARC[1]	Positive n=172			
EIA Repeatedly Reactive	Indeterminate n=0	172	0	0
n=172	Negative n=0	0	0	0
		0	0	0
High Risk[2]	Oisutuve n=176			
EIA Repeatedly Reactive	Indeterminate n=1	176	0	0
n=177	Negative n=0	0	1	0
		0	0	0
High Risk[3]	Positive n=0			
EIA non-reactive	Indeterminate n=28	0	0	0
n=176	Negative n=148	0	8	20
		0	11	137

[1]AIDS n=152 ARC n=20
[2]HIV-1 asymptomatic n=26; prospective high risk n=23; HIV-1 confirmed positives from different geographic locations n=128
[3]Prospective high risk n=176

Additional testing of Genetic Systems ™ HIV-1 western Blot was performed on 333 specimens from persons with clinically conditions unrelated to HIV-1 that might result in a reactivity with proteins present. Though weak bands were occasionally present for viral proteins none of the samples would meet the package insert requirement for an interpretation of POSITIVE.

The AMPLICOR HIV-1 MONITOR® Test, v1.5

LABORATORY PROCEDURE MANUAL

AMPLICOR HIV-1 MONITOR® Test, v1.5	Item No. 21118560018

Laboratory Name
Address
City, State

Director

AMPLICOR HIV-1 MONITOR Test, v1.5
LABORATORY PROCEDURE MANUAL
Human Immunodeficiency Virus Type 1 RNA in Human Plasma

RECORD OF CHANGES: All changes should be made in the space provided or on the corresponding facing page. Each change or notation should be referenced to the appropriate paragraph number and signed by the director.
REVIEW POLICY: This procedure manual is reviewed by the director annually and at other times as required by major changes in procedure or other circumstances affecting laboratory performance of the tests herein described.
NOTE: This Laboratory Procedure Manual contains the most current information available at the time of printing. Refer to the date at the top of the page for the package insert revision used. Therefore, the Laboratory Procedure Manual should not be considered a substitute for the most current revision of the AMPLICOR HIV-1 MONITOR Test, v1.5 package insert. Always refer to the package insert for the most up-to-date information.

PREPARED BY:

Procedure: HIV RNA Standard Version 1.5

Initial Review and Approval		**Date:**
Supervisor		
Technical Specialist		
Lead Medical Technologist		
Lead Medical Technologist		
Director		
Prepared by		

Adopted:		**Retired:**
Reviewed:	**Replaces**	**Date:**

Procedure Locations

Authorized to Approve:
Authorized to Review:

PERSONAL PROTECTIVE EQUIPMENT

Performance of this procedure will expose testing personnel to biohazardous material and possible chemical hazards.

All specimens must be handled as potentially infectious material as outlined in the JHH Safety Manual.

The reagent(s) and/or chemical(s), which are used in this assay, may be hazardous to your health if handled incorrectly. A brief listing of precautions for each chemical hazard is included in the reagent section of this procedure.

More extensive information concerning the safe handling of the reagents and/or chemicals used in this assay, as well as other important safety information, may be obtained by consulting the Material Safety Data Sheet (MSDS) and HAZ-COM Manual, and JHH safety manual. Before performing any part of this assay, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose you to:
[ ] Bloodborne pathogens
[ ] Airborne pathogens
[ ] Hazardous reagents

To perform this procedure, you must use:
[ ] Gloves
[ ] Face shield, or safety glasses with mask
[ ] Laboratory coat
[ ] Gown
[ ] Biological safety cabinet
[ ] Fume hood
[ ] Ventilator
[ ] Disinfectant following procedure:
    [ ] Diluted bleach (1:10 v/v solution made fresh daily)
    [ ] Amphyl
    [ ] Caltech Dispatch with Bleach
    [ ] Miscellaneous, after Hospital Infection Control Department approval

Reference for spill/decontamination:
[ ] MSDS
[ ] Chemical hygiene plan

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1. Principle of Test

General Testing Principle

1.1 The AMPLICOR HIV-1 MONITOR Test, v1.5 is based on five major processes: specimen preparation, reverse transcription to generate cDNA from target HIV-1 RNA and HIV-1 Quantitation Standard (HIV-1 QS) RNA, PCR amplification of target cDNAs using HIV-1 specific primers, hybridization of the amplified cDNAs to target-specific oligonucleotide probes, and colorimetric detection of the probe-bound amplified cDNAs.

The AMPLICOR HIV-1 MONITOR Test, v1.5 permits the simultaneous reverse transcription and PCR amplification of HIV-1 and HIV-1 QS target RNAs. The Master Mix reagent contains a biotinylated primer specific for HIV-1 and QS target nucleic acid and has been developed to yield equivalent quantification of group M subtypes of HIV-1. The performance characteristics of the test with Group O specimens have not been determined. Detection of amplified DNA is performed using target-specific oligonucleotide probes that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon.

The quantitation of HIV-1 viral RNA is performed using the HIV-1 Quantitation Standard (QS). The HIV-1 Quantitation Standard is a non-infectious RNA transcript that contains the identical primer binding sites as the HIV RNA target and a unique probe binding region that allows quantitation standard amplicon to be distinguished from HIV-1 amplicon. The QS is incorporated into each individual specimen at a known copy number and is carried through the five major test processes. HIV-1 RNA levels in the test specimens are determined by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen. The QS compensates for the effects of inhibition and controls for the amplification process to allow the accurate quantitation of HIV-1 RNA in each specimen.

Appropriate selection of primers and probe was critical to the ability of the test to detect the HIV-1 genotype. Accordingly, selection of the target RNA sequence was based on identifying regions within the HIV-1 genome that show maximum sequence conservation among Group M subtypes. The HIV-1 *gag* region encodes the group-specific antigens or core structural proteins of the virion. The AMPLICOR HIV MONITOR Test, v1.5 uses primers SK145 and SKCC1B to amplify an HIV-1 *gag* gene sequence of 155 nucleotides. The nucleotide sequence of primer has been optimized to yield comparable amplification of Group M subtypes of HIV-1.

Specific Test Processes

1.2 Specimen Preparation

Two specimen preparation procedures may be used with the AMPLICOR HIV-1 MONITOR Test, v1.5. This procedure will discuss the standard method. In the Standard Specimen Preparation Procedure, HIV-1 RNA is isolated directly from plasma by lysing virus particles with a chaotropic agent. HIV-1 QS RNA, introduced into each specimen with Lysis Reagent, serves as an extraction and amplification control for each processed specimen. HIV-1 and HIV-1 QS RNAs are precipitated by using alcohol and then resuspended in Specimen Diluent.

1.3 Reverse Transcription and PCR Amplification

Reverse transcription and amplification reactions are performed with the thermostable recombinant enzyme *Thermus thermophilus* DNA Polymerase (r*Tth* pol). In the presence of manganese (Mn^{2+}) and the appropriate buffer, r*Tth* pol has reverse transcriptase and DNA polymerase activities. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

Processed specimens are added to the amplification mixture in amplification tubes (A-tubes) in which reverse transcription and PCR amplification occurs. The AMPLICOR HIV-1 MONITOR test v1.5 uses the primers SK145 and SKCC1B to define a sequence of 155 nucleotides within the highly conserved region of the HIV-1 *gag* gene. The *gag* region encodes the group specific antigens or core structural proteins of the virion. The HIV-1 *gag* genes are generally about 1500 nucleotides in length and are located at the appropriate positions 789-2290 in the HIV genome. The downstream or antisense primer (SKCC1B) and the upstream or sense primer (SK145) are biotinylated at the 5′ends. The reaction mixture is heated in the thermal cycler instrument to allow specific annealing of the downstream primer to target HIV-1 and HIV-1 QS RNAs. In the presence of Mn^{2+} and excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, r*Tth* pol extends the annealed primer to form cDNA.

Target Amplification

Following reverse transcription of target HIV-1 and HIV-1 QS RNAs, the reaction mixture is heated to denature RNA:cDNA hybrids and expose sequences that anneal with the primers. As the mixture cools, the upstream primer (SK145) anneals specifically to the cDNA strand representing each target RNA, r*Tth* pol extends the primer and a second DNA strand is synthesized. This completes the first cycle of PCR, yielding a double-stranded DNA copy of each RNA target region (HIV-1 and HIV-1 QS).

The reaction mixture is heated again to separate the double-stranded DNA and expose the primer-annealing sequences. As the mixture cools, primers SK145 and SKCC1B anneal to target DNA. The r*Tth* pol enzyme, in the presence of Mn^{2+} and excess dNTPs, extends the annealed primers along the target templates to produce a 155-base pair double-stranded DNA "amplicon." The thermal cycler automatically repeats this process for 37 cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the thermal cycler. Amplification occurs only in the region of the HIV-1 genome between the primers; the entire genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the clinical specimen is achieved in the AMPLICOR HIV-1 MONITOR Test, v1.5 by the use of AmpErase® (uracil-N-glycosylase) and deoxyuridine triphosphate (dUTP). AmpErase recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA. It is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains

deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase before the amplification of target DNA. AmpErase, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase is inactive at temperatures above 55°C; therefore, it does not destroy target amplicon during the thermal cycling steps. Following amplification, any residual AmpErase is denatured by the addition of the Denaturation Solution, thereby preventing the degradation of any target amplicon. AmpErase in the AMPLICOR HIV-1 MONITOR Test v1.5 has been demonstrated to inactivate 100 copies of deoxyuridine-containing HIV-1 amplicon per PCR.

1.4 Hybridization Reaction

Following PCR amplification, and the addition of Denaturation Solution to the reaction tubes, the HIV-1 and HIV-1 QS amplicon are chemically denatured to form single-stranded DNA. Aliquots of denatured amplicon are then transferred to separate wells of a microwell plate (MWP) coated with either HIV-1-specific (SK102) or HIV-1 QS-specific (CP35) oligonucleotide probes. The biotin-labeled HIV-1 and HIV-1 QS amplicon are hybridized to the target-specific oligonucleotide probes bound to the wells of the MWP. To achieve quantitative results over a large dynamic range, serial dilutions of the denatured amplicon are analyzed on the MWP.

1.5 Detection Reaction

Following the hybridization reaction, the MWP is washed to remove unbound material and then Avidin-Horseradish Peroxidase Conjugate is added to each well of the MWP. The conjugate binds to the hybridized biotin-labeled HIV-1 and HIV-1 QS amplicons. The MWP is washed again to remove unbound conjugate and then a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to each well. In the presence of hydrogen peroxide, bound horseradish peroxidase catalyzes the oxidation of TMB to form a colored complex. The reaction is stopped by addition of a weak acid and the absorbance of the wells is measured at 450 nm (A_{450}) using a microwell plate reader.

1.6 HIV-1 RNA Quantitation

The quantitation of HIV-1 RNA is achieved by utilizing a second target sequence (HIV-1 Quantitation Standard) that is added to each specimen at a known concentration. The HIV-1 QS is a non-infectious, 233 nucleotide *in vitro* transcribed RNA molecule with primer binding regions identical to those of the HIV-1 target sequence. An amplified product of the same length, 155 bases) and base composition as the target HIV-1 amplicon is generated during the amplification reaction. The probe binding region of the HIV-1 QS amplicon has been modified to differentiate QS amplicon from the HIV-1 target amplicon.

Within the linear range of the assay, the optical density (OD) in each well of the MWP is proportional to the amount of HIV-1 amplicon or HIV-1 QS amplicon in the well. Total OD is calculated by multiplying the OD in each well by the dilution factor for that well. The calculated

total HIV-1 OD or total HIV-1 QS OD is proportional to the amount of HIV-1 RNA or HIV-1 QS RNA, respectively, in each reverse transcription/PCR amplification reaction.

2. Clinical Significance

2.1 Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immunodeficieny Syndrome (AIDS). HIV infection can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to the fetus. Within 3 to 6 weeks of exposure to HIV, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in the peripheral blood. In most infected individuals, this is followed by an HIV-specific immune response and a decline of plasma viremia, usually within 4 to 6 weeks of the onset of symptoms. After seroconversion, infected individuals typically enter a clinically stable, asymptomatic phase that can last for years. The asymptomatic period is characterized by persistent, low-level plasma viremia and a gradual depletion of CD4+ T lympohcytes, leading to severe immunodeficiency, multiple opportunistic infections, malignancies and death. Although virus levels in the peripheral blood are relatively low during the asymptomatic phase of infection, virus replication and clearance appear to be dynamic processes in which high rates of virus production and infection of CD4+ cells are balanced by equally high rates of virus clearance, death of infected cells, and replenishment of CD4+ cells, resulting in relatively stable levels of both plasma viremia and CD4+ cells. Quantitative measurements of HIV viremia in the peripheral blood have shown that higher virus levels may be correlated with increased risk of clinical progression of HIV disease, and that reductions in plasma virus levels may be associated with decreased risk of clinical progression.

2.2 Virus levels in the peripheral blood can be quantitated by measurement of the HIV p24 antigen in serum, by quantitative culture of HIV from plasma, or by direct measurement of viral RNA in plasma using nucleic acid amplification or signal amplification technologies. P24 antigen is the principle core protein of HIV and is found in serum either free or bound by anti-antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), although the usefulness of p24 antigen as a marker of vial load is limited since the antigen is detectable in only 20% of asymptomatic patients and 40-50% of symptomatic patients. Procedures to dissociate antigen-antibody complexes improve the sensitivity of the p24 antigen tests, but the viral protein remains undetectable in most asymptomatic patients. Infectious HIV in plasma can be cultured by inoculation into activated peripheral blood mononuclear cells (PBMC) from normal donors. Quantitation is achieved by inoculating PBMC with serial dilutions of the plasma specimen. Quantitative culture has limited utility for monitoring virus levels in infected individuals since only a small fraction of virus particles is infectious *in vitro*. Infectious virus is often undetectable in asymptomatic individuals. HIV RNA in plasma can be quantitated by nucleic acid amplification technologies, such as Polymerase Chain Reaction (PCR). The AMPLICOR HIV-1 MONITOR Test, v 1.5 is not intended to be used as a screening test for blood or blood products for HIV or as a diagnostic test to confirm the presence of HIV infection. The test is intended for use in conjunction with clinical presentation and other laboratory markers for prognostic assessment of HIV-1 infected patients, and for monitoring the effects of antiretroviral therapy by serial measurements of plasma HIV-1 RNA for patients with baseline viral loads equal to or greater than 25 000 copies of HIV-1 viral RNA/mL.

3. Specimen Requirements

Specimen Collection

Note: Handle all specimens as if they are capable of transmitting infectious agents.

3.1 No special patient preparation before collection is necessary.

3.2 Blood should be collected in sterile collection tubes using EDTA or ACD anticoagulants. The use of ACD anticoagulated specimens will yield results that are approximately 15% lower than test results obtained from EDTA anticoagulated specimens due to the dilution effect of the 1.5ml of ACD anticoagulant present in the blood collection tube.

3.3 Collect one EDTA or one ACD tube using standard venipuncture techniques. Follow the manufacturer's instructions for use of the collection tubes. CSF is also an acceptable specimen.

3.4 **Standard precautions will be observed for the collection, handling, transport, and processing of <u>all</u> specimens**.

Specimen Identification

3.5 Label the specimen container with the patient's name, patient's identification number, history number (if patient is a hospital patient),and the date following the hospital labeling procedures.

3.6 Record the patient's name or identification number, history number, date of birth, specimen collection date and time, and test(s) requested on the requisition,or on an outside client requisition.

3.7 Samples coming for contract work will be labeled as appropriate to the particular study or group. These specimens should come with the appropriate CRF form, and will be entered into LDMS and the proper excel file. (see Importing SOP)

Specimen Transport

3.8 Transportation of whole blood or plasma must comply with country, federal, state and local regulations for the transport of etiologic agents. Whole blood must be transported at 2 -25°C and processed within 72 hours of collection (AACTG as well as the laboratory has documented the lengthened processing time). Plasma may be transported at 2-8°C for 5 days or frozen at -70°C or colder indefinitely.

Specimen Rejection Criteria

3.9 Specimens collected using heparin as the anticoagulant and serum are unsuitable for this test. Note: some research studies may use serum samples, This will be documented to the research protocol team. Some research studies may use heparin samples and the research procedure for Boom extraction should be followed.

3.10 Specimens that are clotted are unsuitable for this test.

3.11 The laboratory has validated the performance of the assay with specimens kept unprocessed up to 72 hours. Specimens that are greater than 72 hours old are unsuitable for this test.

3.12 PPT tubes, effective 4/25/05, are unsuitable per Product notices from Roche.

3.13 Frozen whole blood is unsuitable for the test.

3.14 Inappropriate samples will be canceled in PDS. Attempts will be made to contact the appropriate ordering clinician.

Specimen Handling and Storage

3.15 Separate plasma from whole blood within 72 hours of collection by centrifugation at 800-1600 x g for 20 minutes at room temperature. (See ACTG specific instructions for processing ACTG blood samples. If collected in an EDTA tube for ACTG protocols, then the resulting plasma should then be spun again for an additional 10 minutes for clarification.

3.16 Transfer plasma to a properly identified, sterile, screw-cap, polypropylene tube after centrifugation. If processing for ACTG, transfer to a 15 ml conical tube, centrifuge at 800 x g for 10 minutes and then transfer the plasma to a properly identified, sterile, screw-cap, polypropylene tube.

3.17 Plasma specimens may be stored at 2-8°C for up to 5 days or frozen at -70°C or colder indefinitely. Plasma specimens may be frozen and thawed up to 3 times without a loss of HIV-1 RNA. ACTG samples are kept in the appropriate freezer rack in the upright freezer located in The exact locations can be found in the LDMS storage module.

3.18 Treat all specimens as potentially infectious. Wear gloves and protective clothing and promptly clean up all spills. Dispose of specimens and specimen-containing reagents in marked biohazard containers.

4. Reagents

Reagent Composition

4.1 The AMPLICOR HIV-1 MONITOR Test, v1.5 kit includes the following reagents:

Specimen Preparation Reagents		
HIV-1 MONITOR Lysis Reagent	HIV-1 LYS	Tris-HCl buffer containing 68% guanidine thiocyanate, 3% dithiothreitol, and <1% glycogen
HIV-1 MONITOR Quantitation Standard	HIV-1 QS, v1.5	Tris-HCl buffer containing <0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 primer binding sequences and a unique probe binding region, <0.005% poly rA RNA (synthetic), EDTA,
HIV-1 MONITOR Specimen Diluent	HIV-1 DIL	Tris-HCl buffer containing EDTA, <0.005% poly rA RNA (synthetic), and 0.05% sodium azide

Amplification Reagents		
HIV-1 Master Mix, v 1.5	HIV-1 MMX, v1.5	Bicine buffer containing glycerol, potassium acetate, <0.07% dATP, dCTP, dGTP, dTTP and dUTP, <0.001% SK145 & SKCC1B primers, <0.01% r*Tth* Pol, <0.01% AmpErase® (microbial), and
HIV-1 MONITOR Manganese Solution, v 1.5	HIV-1 Mn^{2+}, v1.5	<2% manganese, acetic acid, amaranth dye and 0.05% sodium azide

Control Reagents		
Negative Human Plasma	NHP	Human plasma, non-reactive by US FDA-licensed tests for antibody to HIV-1 and HIV-2, antibody to HCV, HIV p24 antigen and HBsAg, and 0.1% ProClin® 300
HIV-1 Negative Control	HIV-1 (-)C	Tris-HCl buffer containing <0.005% Poly rA RNA (synthetic), EDTA and 0.05% sodium azide .
External Negative Control	Neg Ct (not in kit)	Known HIV-1 (-) plasma tested and processed in the Blood Bank at JHH
HIV-1 MONITOR Low Positive Control	HIV-1 L(+)C	Tris-HCl buffer containing <0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences, <0.005% poly rA RNA (synthetic), EDTA, and 0.05% sodium azide
AMPLICOR HIV-1 MONITOR High Positive Control	HIV-1 H(+)C	Tris-HCl buffer containing <0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences,

		<0.005% poly rA RNA (synthetic), EDTA, and 0.05%
VQA Control	Log 3 (not in kit)	A solution containing positive control supernatant spiked into seronegative plasma at a concentration of 1500c/mL. Store at –70°C until expiration date which is determined by the VQA lab.

Detection Reagents		
AMPLICOR HIV-1 MONITOR Microwell Plate	HIV-1 MWP	MWP coated with HIV-1-specific DNA probe SK103 (rows A to F) and QS-specific probe CP35 (Rows G and H) Twelve, 8-well strips in one
MONITOR Denaturation Solution	[1] MONITOR DN	1.6% Sodium hydroxide, EDTA, amaranth dye
MONITOR Hybridization Buffer	[2] MONITOR HYB	Sodium phosphate solution containing <0.2% solubilizer and <25% sodium thiocyanate.
Avidin-Horseradish Peroxidase Conjugate	[3] AV-HRP	Tris-HCl buffer containing <0.001% avidin-horseradish peroxidase conjugate, bovine gamma globulin (mammalian), Emulsit 25(Dai-ichi Kogyo Seiyaku Co., Ltd.),
Substrate A	[4A] SUBA	Citrate solution containing 0.01% hydrogen peroxide and 0.1% ProClin 150
Substrate B	[4B] SUBB	0.1% 3,3',5,5'-tetramethylbenzidine (TMB) and 40% dimethylformamide (DMF)
Stop Reagent	[5] STOP	4.9% Sulfuric acid
10X–Wash Concentrate	10X WB	<2% Phosphate buffer, <9% sodium chloride, EDTA, <2% detergent, and 0.5% ProClin 300

Special Supplies

4.2 The following supplies are needed but not supplied in the AMPLICOR HIV-1 MONITOR Test, v 1.5 kit:

Pre-Amplification—Reagent Preparation Area	Disposable nitrile gloves,
	Appropriate hospital lab coats or disposable gowns
	MicroAmp® reaction tubes, tray/retainers and base.
	Plastic resealable bag
	Reservoir (sterile, individually wrapped).
	Cleaning reagents, such as reagent alcohol,isopropanol, and Dispatch.
	Pipettors (capacity 50 & 100 µL) with aerosol barrier or positive displacement Rnase-free tips *Pipettes should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used where specified to prevent specimen and amplicon cross contamination.*
Pre-Amplification—Specimen Preparation Area	Disposable nitrile gloves
	Appropriate hospital lab coats or disposable gowns
	Microcentrifuge (max. RCF 16,000 x g, min. RCF 12,500 x g); Eppendorf 5415C, HERMLE Z230M, or equivalent We currently use the IEC Micromax model.
	2.0 mL and polypropylene screw-cap tubes, sterile, non-siliconized, conical (Sarstedt 72.694.006, or equivalent)
	Tube racks (Sarstedt 93.1428 or equivalent)
	100% ethanol, reagent grade for microbiology or Histology use (freshly diluted to 70% using DEPC grade water). 100% Ethanol,reagent

	grade (University Store cat no. 530891)
	DEPC water Core Store cat. no. 351 068 130
	Isopropanol, reagent grade University Store cat no. 530411
	Fine-tip transfer pipettes, RNase-free.(Fisher cat no. 13-711-28)
	Vortex mixer
	Sterile, disposable, polystyrene serological pipettes (5, 10 and 25 mL)
	Pipettors (capacity 12.5, 25, 100, 200, 400, 600, 800 and 1000 μL) with aerosol barrier or positive displacement RNase-free tips We currently use Rainin brand pipettors. *Pipettes should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used where specified to prevent specimen and amplicon cross contamination.*
	MicroAmp Caps and cap installing tool.

Post Amplification Area—Amplification/Detection	Disposable nitrile gloves
	Appropriate hospital lab coats or disposable gowns
	electronic pipettor (Impact® or AMPLICOR®)
	Aerosol barrier or positive displacement RNase-free tips (25 and 100 µL) and barrier-free tips (100 µL) We currently use Matrix tips (catalog no. 7275). *Pipettes should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used where specified to prevent specimen and amplicon cross contamination*
	Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler
	MicroAmp base and cap installing for use with Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400
	Microwell plate washer (Bio-Tek Instruments ELx50, ELx405, Denley WellWash 4 or equivalent) *Capable of washing 12 x 8 microwell format with 250-300 µL of Wash Solution per well at 30-second time intervals.*
	Microwell plate reader (Coulter UVMax or equivalent) *Specfications: Bandwidth = 10 nm $\pm$ 3 nm; Absorbance range = 0 to $\geq$ 3.00 A_{450}; Repeatability $\leq$ 1%; Accuracy $\leq$ 3% from 0 to 2.00 A_{450}; Drift $\leq$ 0.01 A_{450} per hour.*
	Disposable reagent reservoirs (Denville Cat No. 730-006 or equivalent)
	Microwell plate lid
	Incubator 37°C $\pm$ 2°C
	5 & 10 mL serological pipettes

	Graduated cylinder
	Distilled or deionized water
	Personal Computer with LDMS software
	Reagent for troughs for autoclaving
	CiDecon, Cat. No. 8504
	Dispatch, reorder # 68970)
	Sharps container
	Red lined biohazard boxes

Reagent Labeling and Preparation

4.3 All reagents are labeled by the manufacturer. The labeling includes contents, lot number, expiration date, and storage instructions.

4.4 All reagents are liquid, ready-to-use. Working reagents are prepared at specific intervals during the test process. These are:

- Working Master Mix: Prepare by adding 100 µL HIV-1 Mn^{2+} to one vial HIV-1 MMX, v1.5, store at 2–8°C, use within 4 hours of preparation. Quantity sufficient for 12 reactions.
- Working Lysis Reagent: Prepare by adding 100 µL HIV-1 QS, v1.5 (Standard Specimen Preparation) to 1 bottle HIV-1 LYS, store at room temperature, use within 4 hours of preparation. This solution must be thawed prior to use. There should be no crystals present before adding the HIV-1 QS solution.
- Working Substrate: Prepare by adding 3 mL [4B] SUB B to 12 mL [4A] SUB A; stable for 3 hours; protect from direct light and exposure to metals or oxidizing agents. **This is a 1:5 ratio. The demarcations on the 15ml conical tubes are not exact. It is best to measure out the volumes using serological pipettes.**
- Working Wash Solution: Prepare by adding 1 volume 10X-WB to 9 volumes distilled or deionized water; stable for 2 weeks; store at 2–25°C in a clean, closed plastic container. This container should be labeled with preparer's initials and correct expiration date.

Reagent Storage and Use

4.5 Store the following reagents at 2–8°C: HIV-1 LYS; HIV-1 QS, v1.5; HIV-1 DIL ; HIV-1MMX, v1.5; HIV-1 Mn^{2+}, v1.5; NHP; HIV-1 (-)C; HIV-1 L(+)C; HIV-1 H(+)C; HIV-1 MWP; [3]AV-HRP; [4A] SUB A and [4B] SUB B.

Store the following reagents at 2-25°C: [1] MONITOR DN, [2] MONITOR HYB, [5] STOP and 10X WB.

Do not freeze reagents.

4.6 Do not use reagents beyond the expiration dates shown on the packages. All reagents are stable to the expiration date.

The [3] AV-HRP, [4A] SUB A and [4B] SUB B are stable for 3 months or the expiration date, whichever comes first, at 2–8°C once opened.

The HIV-1 MWP is stable for 3 months or until the expiration date, whichever comes first, at 2–8°C once opened. Store MWP in a resealable pouch containing desiccant.
The stability of the Working Reagents is noted with their instructions for preparation—See Section 4.4.

4.7 Do not mix reagents from different lot numbers. Do not pool reagents. Dispose of unused reagents and waste in accordance with all local, country, state, and federal regulations.

4.8 Handle all reagents with caution and avoid contact with skin, eyes, or mouth. Refer to the package insert for any known toxicity.

4.8.1 These reagents contain **sodium azide**: HIV-1 QS, v1.5; HIV-1 MMX, v1.5; HIV-1 Mn^{2+}, v1.5; HIV-1 DIL; HIV-1 (-) C; HIV-1 L(+)C; HIV-1 H(+)C. Avoid swallowing and contact with skin and mucous membranes. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large amount of water to prevent azide build-up.

4.8.2 These reagents contain **dimethylformamide**: [4B] SUB B and Working Substrate. Dimethylformamide is an irritant and has been reported to be teratogenic in high oral doses. Wear gloves when handling these reagents. Avoid skin contact, inhalation of fumes, and ingestion. May cause harm to the unborn child.

4.8.3 These reagents carry a **burn warning**: Denaturation and Stop Solutions. Avoid contact of these materials with the skin, eyes, and mucous membranes. If contact does occur, immediately wash with large amounts of water. If spills of these reagents occur, dilute with water before wiping dry.

4.8.4 These reagents are marked as **harmful or irritants**: HIV-1 LYS and [2] MONITOR HYB. Avoid contact of these materials with the skin, eyes, and mucous membranes. If contact does occur, immediately wash with large amounts of water.

4.9 Reagents required to perform this assay are located:

The kits are kept in their packing boxes in the cold room. When received the boxes are labeled with the received date. The boxes are numbered. Once the kits are validated, the entire kit will remain in the cold room. When a new packing box is opened, the kits in the box will also be labeled in numerical order within a packing box. The extraction and amplification reagents are removed only when needed, this is usually right prior to the start of a run. The technologist will remove the extraction and amplification reagents from the whole kit before proceeding to the extraction. The technologist will label the kits in use with their initials, and they will record the number of the kits in use on the viral load worksheet. The kits are on a blanket order with Roche notifying the supervisor of the lot number to be shipped along with the ship date. Typical orders are of 160 kits/month. The alcohols are kept in the flammable cabinet in, with extras kept in the flammable cabinet in. When the second to last bottle is removed from, a note is left in the order notebook The wash buffer is removed from the kit and put on the shelves in. The shelves should be labeled with the proper kit information. Distilled, deionized water is retrieved from and placed into containers for use in

5. Test Procedure

Run Size and Workflow

5.1 Each kit contains sufficient reagents for two 12-specimen runs. The Specimen Preparation Reagents and Amplification Reagents are packaged in 12-test, single-use bottles. Therefore, it is recommended that processing should be in batches of multiples of 12 for the most efficient use of reagents, specimens, and controls. In this laboratory, assays are typically performed in batches of 3 plates. If the volume is down, then 2 plates or less are run. If a technologist works 10 hour shifts, then he/she may perform 4 plates.

Examine all reagents for sufficient volume before beginning the test process. (See Section 4.7.)

5.2 The AMPLICOR HIV-1 MONITOR Test, v1.5 may be completed in 1 day or over 2 days. Testing can be completed over 2 days, by starting Specimen and Control Preparation on day 1 and following with reagent preparation, reverse transcription, amplification and detection on day 2. However in this laboratory assays are typically finished in one day with few exceptions.

Reagent Preparation—Part A-Area 1: Pre-Amplification

5.3 The Amplification Reagents must be at room temperature before beginning test procedure.

Wear new gloves and a lab coat to perform the following steps:

A.1 Determine the number of reaction tubes needed for patient specimen and control testing. Place the tubes in the MicroAmp tray and lock into place with retainer.

A.2 Prepare the Working Master Mix: Add 100 µL of HIV-1 Mn^{2+}, v1.5 to one vial of HIV-1 MMX, v1.5. Recap and mix well by inverting 10–15 times. *Note pink color that confirms that manganese has been added to Master Mix.* This mixture is sufficient for 12 reactions and stable for 4 hours. Discard remaining Manganese Solution. *Do not vortex the working master mix.*

A.3 Pipette 50 µL of Working Master Mix into each reaction tube using a pipettor with an aerosol barrier or positive displacement tip. *Visually inspect the tubes for the pink color to confirm that the Working Master Mix was added.* Do not cap the reaction tubes at this time.

A.4 Place the tray and appropriate number of reaction tube caps in a resealable plastic bag and seal securely. Remove and dispose of gloves. Remove labcoat and leave it in Area 1 – Reagent Preparation Area. Area 1 is room 304 of the HIV Specialty Laboratory.

A.5 Store sealed bag and tray at 2-8°C in Area 2—Specimen Preparation Area—until ready to add samples. Area 2 is room 308 of the HIV Specialty Laboratory.

Important Note: Amplification must begin within 4 hours of preparing the Working Master Mix.

A.6 The order in which specimens are prioritized are as follows: The technologist must check the untested hospital box for any pending hospital specimens first. A pending log must be printed from the PDS lab computer system. This pending must be compared with the actual specimens. To generate a pending log one

must log onto the PDS system. Enter CLI for clinical at the Pathology prompt, enter RPT for reports at the clinical prompt, enter P for pending, enter W for workstation, and enter HIVSP. At this point you can select which assay is being performed and which one that you need a pending log for. The second choice of specimens should be the repeat/dilution folder. The third choice of specimens should be any Standard assay contract samples. The "to do" list posted in Pathology 313 should be checked to see what protocols are standard and what specimens are outstanding.

NOTE: The Standard (Section 5.4) Specimen Preparation methods may be used with the AMPLICOR` HIV-1 MONITOR Test, v 1.5 kit.

Standard Specimen Preparation—Part B—Area 2: PreAmplification-Specimen Preparation Area

5.4 The Specimen Preparation and Control Reagents must be at room temperature before beginning procedure.
Specimens must be at ambient temperature before use. If using frozen specimens, thaw at room temperature before use.

Use only screw-cap tubes for specimen preparation to prevent splashing and potential cross-contamination of specimens. *Do not use snap-cap tubes.*

A precipitate forms in HIV-1 LYS upon storage at 2-8°C. Dissolve the precipitate by warming at 25–37°C for a maximum of 30 minutes and mixing thoroughly. Do not place in an incubator or water bath to thaw.

Prepare a viral load worksheet (see appendix). Record the kit received date, packing box and kit number.

Wear new gloves and a new lab coat to perform the following steps:

B.1 Prepare 70% ethanol: For one plate mix 10.5 ml of 100% Ethyl Alcohol and 4.5 ml of DEPC water. Be sure to label alcohol bottle with an open date.

For two or three plates, double or triple the volumes respectively. There is a recipe chart in the pre-amplification room on top of the flammable cabinet.

B.2 Prepare Working Lysis Reagent: Vortex HIV-1 QS, v1.5 for 3-5 seconds. Add 100 µL HIV-1 QS, v1.5 to one vial of HIV-1 LYS, v1.5. Recap and mix well. Discard remaining HIV-1 QS, v1.5.

Important Note: The Working Lysis Reagent must be used within 4 hours of preparation.

B.3 Label a 2 mL screw-cap tube for each specimen and control. Place an orientation mark on each tube.

B.4 Vortex specimens for 3–5 seconds.

B.5 Add 600 µL Working Lysis Reagent to each labeled tube.

B.6 Vortex NHP for 3–5 seconds. Add 200 µL NHP to each control tube containing Working Lysis Reagent. Cap the tubes and vortex for 3-5 seconds.

B.7 Vortex HIV-1 (-)C, HIV-1 L(+)C, and HIV-1 H(+)C for 3-5 seconds. Add 50 µL of HIV-1 (-)C, HIV-1 L(+)C, and HIV-1 H(+)C to the appropriate tubes. Cap tubes and vortex for 3-5 seconds.

B.8 Add 200 µL of each specimen to the appropriately labeled tube containing Working Lysis Reagent. Cap the tubes and vortex for 3-5 seconds.

B.9 Incubate specimen and control tubes for 10 minutes at room temperature.

B.10 Add 800 µL 100% isopropanol to each tube. Cap tubes and vortex immediately for 10 seconds.

B.11 Place tubes in microcentrifuge with orientation mark facing outward. Centrifuge for 15 minutes at 12 500-16 000 x g at room temperature.

B.12 Using a new fine-tip transfer pipette for each tube, carefully aspirate and discard the supernatant without disturbing the pellet (which may not be visible).

B.13 Add 1.0 mL 70% ethanol to each tube. Cap tubes and vortex for 10 seconds.

B.14 Place tubes in microcentrifuge with the orientation mark facing outward. Centrifuge for 5 minutes at 12 500–16 000 x g at room temperature.

B.15 Using a new fine-tip transfer pipette for each tube, carefully aspirate and discard the supernatant without disturbing the pellet. The pellet should be clearly visible now.

Important Note: Residual supernatant can inhibit the amplification.

B.16 Add 400 µL HIV-1 DIL to each tube. Cap tubes and vortex vigorously for 10 seconds. Some insoluble material may remain.

Specimens must be amplified within 2 hours of this point. If proceeding, continue with step B.17. If not, store specimens frozen at -20°C or colder for up to 1 week with no more than one freeze thaw.

B.17 Remove MicroAmp tray from the refrigerator. Using a pipettor fitted with a new aerosol barrier or positive displacement tip for each, add 50 µL of each processed control and specimen to the appropriate reaction tube containing Working Master Mix. Be careful to avoid transferring any precipitated material that may not have gone back into solution.

B.18 Cap the tubes. Record positions of controls and specimens in the MicroAmp tray. Reverse transcription and amplification must be started within 45 minutes of this point.

B.19 Transfer the MicroAmp tray with the sealed tubes to the Amplification/Detection Area. This will be Pathology room 311. Lab coats and/or any equipment used in the pre-amplification area should remain in that area only. Lab coats used in Path 308 cannot be worn outside of Path 308.

B.20 Decontaminate work area **with the appropriate cleaning material for the dead air box being used. For example, decontaminate with Dispatch™. An alternative cleaning solution would include a 1:10 dilution of bleach followed by cleaning area with propanol. Turn on the UV light and record on the appropriate hood maintenance worksheet.**

B.21 Record which hood was used. There is a space on the viral load worksheet for recording the specific hood # that was used during extraction.

Reverse Transcription and Amplification – Part C – Area 3: Post-Amplification/Detection Area

5.6 Turn on the GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler at least 30 minutes before use.

Perform the following steps:

C.1 Place the MicroAmp tray/retainer assembly into the thermal cycler block.

C.2 Program the Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler as follows:

HOLD Program	2 minutes at 50°C
HOLD Program	30 minutes at 60°C
CYCLE Program (8 Cycles)	10 seconds at 95°C, 10 seconds at 52°C, 10 seconds at 72°C
CYCLE Program (23 Cycles)	10 seconds at 90°C, 10 seconds at 55°C, 10 seconds at 72°C
HOLD Program	15 minutes at 72°C

Within the CYCLE programs, the ramp times and allowed setpoint error should be left at the default settings: ramp time = 0.00, setpoint error = 2°C.

Link the 4 programs together into a METHOD program.

Consult the Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 User's Manual for additional information on programming and operation of the thermal cycler.

C.3 Start the METHOD program. The program runs approximately 1 hour and 30 minutes. Each thermocycler has the method program number listed on the instrument as well as in the maintenance manual.

C.4 Remove the tray from the thermal cycler at any time during the final HOLD program, place in the MicroAmp Base and continue immediately with step C.5.

Do not allow the reaction tubes to remain in the thermal cycler beyond the end of the final HOLD program, and do not extend the final HOLD program beyond 15 minutes.

DO NOT BRING AMPLIFIED SAMPLES INTO THE PRE-AMPLIFICATION AREA. AMPLIFIED CONTROLS AND SAMPLES SHOULD BE CONSIDERED A SOURCE OF POTENTIAL CONTAMINATION.

C.5 Remove the caps from the reaction tubes carefully to avoid creating aerosols of the amplification products. **Immediately** pipette 100 µL **[1] MONITOR DN** to each reaction tube using a multichannel pipettor, with aerosol barrier tips, and mix by pipetting up and down 5 times.

The denatured amplicon can be held at room temperature for no more than 2 hours before proceeding to Detection (Part E). If the detection reaction cannot be performed within 2 hours, caps the tubes with new caps and store the denatured amplicon at 2–8 ℃ for up to 1 week

C.6 Perform a run history on the thermocycler to ensure proper thermocycler function. Record the run history information in the thermocylcer maintenance book.

C.7 Record the thermocycler information on the viral load worksheet.

Detection – Part D – Area 3: Amplification/Detection Area

5.7 Allow all reagents to warm to room temperature before use. Allow the HIV-1 MONITOR MWP to warm to room temperature before removing from the foil pouch.

Examine the 10X Wash Concentrate for any precipitate. If precipitate exists, warm to 30–37°C to dissolve.

Perform the following steps:

D.1 Prepare the Working Wash Solution: Add 1 volume 10X WB to 9 volumes of distilled or deionized water and mix well. Store at 2–25°C, in a clean, closed plastic container. Solution is stable for 2 weeks. Be sure to label the container with preparer's initials and the correct expiration date.

D.2 Pipette 100 µL [2] MONITOR HYB to each well of the MWP.

If the denatured amplicon were stored at 2–8 ℃, incubate at 37 ℃ for 2–4 minutes in order to reduce viscosity.

D.3 Rows A-F of the MWP are coated with HIV-1-specific oligonucleotide probe. Rows G & H are coated with HIV-1 QS-specific oligonucleotide probe. Using a Matrix 12-channel pipettor and aerosol barrier tips, add 25 µL of denatured amplicon to the HIV-1 wells in row A of the MWP and mix up and down 10 times. Make 5-fold dilutions in the HIV-1 wells in rows B-F as follows. Transfer

25 µL from row A to row B and mix as before. Continue through row F. Discard 25 µL from row F along with pipette tips.

D.4 Using a Matrix 12-channel pipettor and aerosol barrier tips add 25 µL of denatured amplicon into the HIV-1 QS wells in row G of the MWP and mix up and down 10 times. Transfer 25 µL from row G to row H. Mix as before and discard 25 µL from row H.

D.5 Cover the MWP with the MWP lid and incubate for 1 hour at 37°C ± 2°C.

D.6 Wash the MWP with the Working Wash Solution using an automated MWP washer.

For automated washing:

ELx405:
To PRIME washer:
Run
Prime
Select Prime Program 08 AMPLICOR
Press enter
Screen will say "Connect Reagent Bottle"
Press start
Repeat prime by pressing enter
@Prime Program 08 press enter
@Connect Reagent Bottle press start

To Begin WASH Cycle:
Main Menu
Run
Wash
Scroll through OPTIONS until screen says "Wash Program 05, AMPLICOR"
Enter
Press start

ELx50 "E"
To PRIME Washer:
Run
Prime
Scroll through OPTIONS until screen says "New Buffer Prime Program 02"
Enter
Start

To Begin WASH Cycle
Main Menu
Run
Wash
Scroll through OPTIONS until screen says "Wash Program 05 AMPLICOR"

Enter
@Number of Strips enter the # of strips to be washed
Enter
Start

ELx50 "C"
To Prime Washer: Same as ELx50 "E"
To Begin Wash Cycle:
Maine Menu
Run
Wash
Scroll through OPTIONS until screen says "Wash Program 02 AMPLICOR"
@ Number of Strips enter the # of strips to be washed
Enter

Fill each well to top with Working Wash Solution (approximately 250–300 µL), soak for 30 seconds and aspirate dry.

Repeat step b four additional times.

After automated washing is complete, tap the plate dry.

D.7 Add 100 µL [3] AV-HRP to each well.

D.8 Cover the MWP and incubate for 15 minutes at 37°C ± 2°C.

D.9 Prepare Working Substrate by mixing 12.0 mL [4A] SUB A and 3 mL [4B] SUB B. Store at room temperature and protect from exposure to direct light. This is a 1:4 ratio. The demarcations on the 15ml conical tubes are not exact. It is best to measure out the volumes using serological pipettes.

Prepare Working Substrate no more than 3 hours before use.

D.10 Wash the MWP as described in E.6, there is no need to prime the washer again. It is already primed.

D.11 Add 100 µL Working Substrate to each well.

D.12 Allow color to develop for 10 minutes at room temperature (20–25°C) in the dark.

D.13 Add 100 µL [5] STOP to each well.

D.14 Measure the optical density at 450 nm within 10 minutes of adding the [5] STOP.

D.15. Turn on the printer and plate reader (BioTek ELX 800) at least 10 minutes before reading to ensure that the plate reader is warmed up.

D.16 Once a shift the self-test is performed (by the first person on the shift):

Reader does a self test

After completed, at main menu press button under UTIL.

Next Press button under TESTS

Next Press button under SYS

Reader will perform self test and print out report

Check the report to see that it states that the System Test Passed (on the bottom of the report).

Initial the print out and maintenance sheet in plate reader QC manual and place print-out in the manual.

D.17 Perform autocal

At main menu press arrow/button under READ.

Press Enter Wavelength: Single will appear

Press Enter

Meas:450 will appear.

Press enter

Number of Samples: 96 will appear

Press enter.

Place plate in carrier – Ignore this command and Press enter

Reader will perform autocal and print out report.

Initial maintenance sheet and initial report. Place print out in manual

D.18 Read Plate

Turn on computer attached to plate reader, if not on all ready.

Open Windows.

Double click on MSDOS desktop icon

Click Ok to current default device is Biotek ELx800

(current default configuration is <default>)

Place disk in drive A

Place plate in carrier

ALT – R – use mouse or tab key to get in window.

Enter run ID, sequence number (plate number)

Tab to Drive A, click ok

Plate will be read

On screen will state: Biotek Elx800 read successfully. Output is store in A;\Devdata:run ID

Click Ok

Check ODs on screen with how plate looks visibly.

D.19 Switch the wash tubing from the wash bottle to the DI water bottle.
Main menu screen press "MAINT" then press start. This will rinse the wash lines free of residual wash solution preventing wash crystal buildup.

D.20 Record washer maintenance.

D.21 Record the specific Matrix pipettor used on the viral load worksheet.

D.22 Return ACTG samples to their appropriate freezer locations. The lab validated the long term storage at approximately -30°C. At present, tested samples are stored for 6 months. If the sample needs to be repeated, place back in the untested box.

6. Calculations

6.1 Results of the AMPLICOR HIV-1 MONITOR Test, v1.5 are reported in copies/mL For each specimen and control, calculate the HIV-1 RNA level as follows :

A.1 The HIV-1 wells in rows A through F represent neat and 1:5, 1:25, 1:125, 1:625 and 1:3125 serial dilutions, respectively, of the HIV-1 amplicon. The absorbance values should decrease with the serial dilutions, with the highest OD_{450} for each specimen and control in row A and the lowest in row F.

A.2 Choose the well with the lowest OD_{450} between 0.20 and 2.0 OD units.

A.3 If any of the following conditions exist, see Interpretation of tests with out-of-range or out-of-sequence OD values.

- All HIV-1 OD values <0.20
- All HIV-1 OD values >2.0
- HIV-1 OD values do not decrease from well A to well F (out-of-sequence)

A.4 Subtract background OD, 0.070, from the selected HIV-1 OD value.

A.5 Calculate the total HIV-1 OD by multiplying the background-corrected OD value, of the selected HIV-1 well, by the dilution factor associated with that well.

Row	Dilution Factor
A	1
B	5
C	25
D	125
E	625
F	3125

A.6 The Quantitation Standard wells in rows G and H represent neat and 1:5 dilutions, respectively, of the QS amplicon. The absorbance values should decrease with the serial dilutions, with the highest OD_{450} for each specimen and control in row G and the lowest in row H.

A.7 Choose the well with the lowest OD_{450} that is between 0.30 and 2.0 OD units.

A.8 If any of the following conditions exist, see interpretation of tests with out-of-range or out-of-sequence OD values.

- Both QS OD values <0.3
- Both QS OD values >2.0
- QS OD values out of sequence, row H higher than row G.

Note: All patients specimens and controls should yield QS OD values that meet the criteria described in Steps A.6 – A.8, demonstrating that the specimen processing, reverse transcription, amplification, and detection steps were performed correctly. If any control has a QS OD value that does not meet the criteria described in Steps A.6 – A.8, the entire run is invalid. If any specimen has a QS OD value that does not meet the criteria described in Steps A.6 – A.8, the result for that specimen is invalid, but the run is still acceptable provided the controls have QS OD values that meet the criteria described in Steps A.6 – A.8.

A.9 Subtract background OD, 0.070, from the selected QS OD values.

A.10 Calculate the Total QS OD by multiplying the background-corrected OD value of the selected QS well by the dilution factor associated with that well.

Row	Dilution Factor
G	1
H	5

A.11 Calculate the HIV-1 RNA copies/mL as follows:

HIV RNA Copies/mL =

Total HIV OD/Total QS OD x Input QS Copies/PCR x Dilution Factor

Total HIV OD: calculated using Steps A.1 – A.5

Total QS OD: calculated using Steps A.6 – A.10

Input QS Copies/PCR: specific to each lot of QS, see the AMPLICOR HIV-1 MONITOR Test, v1.5 data card.

Dilution Factor: factor to convert copies per PCR to copies per mL of plasma.

Dilution Factor = 40 for the Standard Specimen Preparation

Computerized (Manual – LDMS) Calculations

Standard Roche. Use Roche Amplicor (Kit Controls) template.

1) Kit controls are pre-loaded. You cannot modify/delete controls on templates.
2) The LDMS will automatically re-distribute the controls as specimens are added past plate one.
3) The VQA 1500cp/mL copy control will automatically be added to all plates past plate 3 as needed.
4) Invalid controls may be re-detected If they are dilutional errors.
5) External negative controls must be manually added to each plate.

B.1 On LDMS System, go to the assay module.
B.2 Click on the (+) sign next to Viral Load RNA from the Assay lists box.
B.3 Then click on the New Run/Not Setup button on upper right side of Search Criteria box.
B.4 Click on the Select button at the bottom of the Assay Selection screen. The Filters/Criteria screen appears.
B.5 At this time it is unclear whether the Filers/Criteria screen will be used. This section is used to pull pending viral loads from the database by using specific parameters. . At this point we will not be using the button "All Pending Specimens" because the list pulled would be too numerous to look through.
The best suggestion at present to find specimens is to select the group from the Group combo box. Then narrow your search, create query statements using the Field, Operator and Value combo boxes.
B.6 Click the Find Specimens button at the bottom of the screen. This will take you to the Specimens Found screen. Use the shift or ctrl keys to select each specimen you want to place in the assay or use the buttons at the bottom of the Specimens Found screen.
B.7 After selecting specimens, click the Add to Plate button at the bottom of the screen. LDMS will automatically move to the Plate Preview screen and load the chosen specimens onto the plate in the order determined by the user.
B.8 **You can also upload specimens using the options button from a text list or individually add the samples by typing them in and adding them to the run. For any non logged (non ACTG samples), use the red plus sign to add the samples to the run.**
B.9 Repeat the process from step five if more than one group needs to be put on the run.
B.10 In the Plate Preview screen the user can add and delete plates to run; move, delete add and modify specimens; move, add, and modify controls; save the template to run later; click the Run Now button to run the assay.
B.11 After saving the assay optical densities from the LDMS Remote Reader Software, it can be read into the LDMS database and merged with the template previously set up. Put the disk into the A: drive. Go to the Assay module again.
B.12 Click the Viral Load RNA.
B.13 Click on the Runs Not Performed button.
B.14 Enter the run id of your assay in the Run Id field.

B.15 Click on your assay to select it, then click on the Select button at the bottom right of screen. The Preview tab will activate.
B.16 Click on the Preview tab, then click the Run Now button the bottom of the screen.
B.17 The kit control field box will appear. Select the correct kit and the control lot numbers and ranges will be uploaded. Enter tech intials and dates for each step of the assay.
B.18 A file dialog box will appear. Open the A:/devdata folder, then click on your file.
B.19 Click on the Open button on the File dialog box, and LDMS will begin reading the raw data from the disk in your A: drive.
B.20 When the LDMS is finished reading the assay data from your remote reader disk, the Results screen will appear in Plate Results view displaying the raw data from your remote assay run.
B.21 Click on the Calculated Results button to view results on a specimen by specimen basis.

7. Reporting Results

Reporting Format

Results of the AMPLICOR HIV-1 MONITOR Test, v1.5 are reported in HIV-1 RNA copies/mL. The run must be QAed prior to results being released. Hospital results are reported in the. The following steps should be followed for reporting hospital results:

1. For ACTG or industry samples, print out clinical reports of all valid results on the run. Match the clinical report with the appropriate fax cover sheets. Fill in the appropriate information on the fax cover sheets. After the run has been QAed, the administrative assistant will fax the results to the sites.

7.2 Interpretation of tests with out-of-range or out-of-sequence OD values

- All HIV-1 OD values <0.20. Do not calculate these results. Report as "HIV-1 RNA Not Detected less than 400 copies/mL" (for Standard specimen preparation procedure). Samples should be run 2 times to see if a result can be obtained. If no result is obtainable, then the result should read that "results were not obtainable". No further testing should be done. The boom assay is no longer being performed.
- All HIV-1 OD values >2.0. The HIV-1 copy number is above the linear range of the assay. Report the result as "Not Determined". If performing the Standard specimen preparation procedure, prepare a 1:100 dilution of the original plasma sample with HIV- negative human plasma and repeat the test. Calculate the results as above then multiply the final result by 100.
- HIV-1 OD values out of sequence. If the HIV-1 wells do not follow the general pattern of decreasing OD values from well A to well F, an error in dilution may have occurred. Examine the data using the criteria shown below in examples of out-of-sequence OD values to determine if an error occurred. If an error occurred, the result for that specimen is invalid and the entire test procedure for that specimen should be repeated.

- All QS OD values <0.30. Either the processed specimen was inhibitory to amplification or the RNA was not recovered during specimen processing. The result for that specimen is invalid. Repeat the entire test procedure for that specimen.
- All QS OD values >2.0. An error occurred. The result for that specimen is invalid. Repeat the entire test procedure for that specimen.
- QS OD values out of sequence. If the QS wells do not follow the general pattern of decreasing OD values from well G to well H, an error occurred. The result for that specimen is invalid. Repeat the entire test procedure for that specimen.

Examples of out-of-sequence OD values

In reactions containing high HIV-1 RNA copies/mL, wells A, B and C can become saturated, turning a greenish-brown color before the addition of Stop Reagent and a brown color after, resulting in lower OD_{450} values (see examples 1 and 2 in Table 1 below). These results are valid.

In reactions containing low HIV-1 RNA copies/mL, wells B through F may contain background OD values (see example 3 in Table 1 below). These results are valid.

All wells with OD values $\leq$2.0 and $\geq$0.20 should follow a pattern of decreasing OD values from well A to well F. If these wells do not follow a pattern of decreasing OD values, an error occurred. The result for such a specimen is invalid. Repeat the entire test procedure for that specimen. In some instances this specimen can be redetected. Sometimes an out of sequence may have occurred. When an out of sequence has occurred, that specimens' well can be reselected. The LDMS will ask for documentation as to why the well was reselected.

Table 1: Sample Results OD_{450} nm

Row	Dilution Factor	Example 1	Example 2	Example 3	Example 4
A	1	2.610	2.564	0.812	3.126
B	5	2.461	2.684	0.161	0.857
C	25	3.112	2.432	0.055	1.432
D	125	2.668	1.032	0.064	0.292
E	625	2.948	0.287	0.079	0.074
F	3125	1.568	0.074	0.052	0.066
Interpretation		Very high titer specimen. Not an error.	High titer specimen. Not an error.	Low titer specimen. Not an error.	Error.

Expected Values

7.3 After diagnosis, a baseline viral load should be done. Viral load levels can range from less than 50 copies/mL to more than 20 million copies/mL. The viral load should be measured at regular intervals afterward to aid the physician in making appropriate treatment decisions.

If viral load levels remain consistently low, <10 000 copies/mL, the decision to start taking HIV-1 medication may be delayed. With increasing or high viral load levels, >55 000 copies/mL, highly active antiretroviral therapy may be advised.

Increases or decreases in viral load of more than three-fold may be considered meaningful. If viral load has not decreased by at least three-fold after initial therapy, or if viral load goes up by more than three-fold at any time during therapy, the patient should consult with their physician.

Effective treatment should result in large reductions in viral load, 1 log by 8 weeks, and continue to reduce viral load until it is below 50 copies/mL

8. Quality Control

Quality Control Information

8.1 At least one replicate of the AMPLICOR HIV-1 MONITOR (-) Control, the AMPLICOR HIV-1 MONITOR Low (+) Control, and the AMPLICOR HIV-1 MONITOR High (+) Control must be included in each test run. Specimens and controls from separate specimen preparation batches may be amplified and detected at the same time. However, each separate specimen batch is validated individually by the set of controls included with the batch. Therefore, it is possible to reject one batch of specimens from a common amplification and/or detection run while accepting another batch upon the performance of the controls processed with those specimens.

All test specimens and controls prepared in the same batch should be amplified and detected in adjacent positions in the thermal cycler and on the detection plate. The exact order or placement of these specimens and controls in the thermal cycler or detection plate is not critical.

Control Material Preparation and Storage

8.2 Three controls are provided for use: AMPLICOR HIV-1 MONITOR (-) Control, AMPLICOR HIV-1 MONITOR Low (+) Control, and AMPLICOR HIV-1 MONITOR High (+) Control. The controls are liquid, ready to use.
Store the controls at 2–8°C. The products are stable until the expiration date.
Refer to Sections 5.4 and 5.5 for instructions on the preparation of Controls.
If 4 plates of specimens are prepared , then an additional external control is used. The VQA control, log 3, will automatically be added to the 4th plate.
An external negative control should be added to each plate. The negative human plasma included in the kit can be used as the external negative control.

8.3 The quality control material is located:

The kit controls are stored inside the kit which is kept in the walk- in refrigerator in The VQA controls are kept in the VQA rack in the appropriate freezer in

Recording Quality Control Results

8.4 The Quality Control results are recorded in the Excel file Additional controls may be added based on direction from the supervisor or lab director.

8.5 Any technical delays associated with Quality Control Failure or dilution error is recorded in the QA Manual volume 2 under technical delays.

Acceptable Limits

8.5 The AMPLICOR HIV -1(-) Control should yield all HIV-1 OD values <0.20.
The assigned range for the AMPLICOR HIV-1 MONITOR Low (+) and High (+) Controls is specific for each lot of control, based upon the specimen preparation method utilized and is provided on the AMPLICOR HIV-1 MONITOR Test, v1.5 Data Card supplied with the kit.
The HIV-1 RNA copies/mL for both positive controls should fall within the range indicated on the Data Card.
All controls should yield QS OD values that meet the criteria described in the RESULTS section, demonstrating that the control preparation, reverse transcription, amplification, and detection steps were performed correctly.
This range is to be used until at least 20 to 30 values have been run and an in-laboratory range can be established. These ranges should be checked periodically and any trends noted.
The kit information is stored in the LDMS. The ranges of the kit controls are programmed into the LDMS. These ranges will be used to validate the run. In house calculated ranges of for internal purposes.
The negative control and external negative controls should yield a "not detected" result; i.e. all HIV OD values less than 0.200.
The LDMS system will validate the assay based on the following:
Basic Criteria to define a valid run for the Roche HIV-1 Monitor™ Test

4.

1. All kit copy control results must be valid
   a. no out of sequence errors in the 0.2-2.0 working OD range
   b. QS result must be valid- OD must be between 0.3-2.0 in 1:1 or 1:5 dilution
   c. No OD ratio failures (run may be considered valid after re-detection of control)
   d. Any failure of the control criteria results in an invalid run. If the controls resulted in a D censor, then re-detection of one or all controls may validate a run, otherwise, all samples and controls need to be re-extracted, re-amplified, and re-detected. If controls upon redetection still do not fall within range, then the entire run should be repeated and censored as a K kit control problem.
2. All Kit positive control results must be within the range provided by the manufacturer
   a. if outside the manufacturer range, all samples within the run need to be repeated
   b. control can be re-detected in cases where applicable
3. Kit negative control must be undetectable- all WT ODs <0.2
4. Any extra kit controls must be within range. Any external negative controls must be negative. However, if an external control is a dropped pellet then the run is still acceptable. If

more than one of the external negative controls is a dropped pellet, the run will be censored as K and repeated. In addition, any extra kit controls that need to be redetected must fall within range after the redetection. If they do not, the run is repeated and censored as a K kit control problem.
Validity criteria have been established using the Roche HIV-1 Monitor™ package insert as well as VQA data. VQA analyses demonstrate that the out of sequence rule should only be applied to ODs that fall within the 0.2-2.0 working range. Additional studies demonstrate that the OD ratio rule can predict accuracy in estimations of RNA recovery. The OD ratio may be obtained in situations where there are two or more WT ODs between 0.2-2.0. The background OD (0.07) is first subtracted from the OD values, and then a ratio of the higher OD to the lower OD is determined. The OD ratio is deemed acceptable if the OD ratio is between 3-7 fold. VQA analyses demonstrate that OD ratios that fall outside this range may result in an inaccurate estimation of RNA copy number.

It is required that the results be reviewed and QAed by the supervisor, director, or designee before they are released.
The VQA panel of 0, log 3, log 4 and log 5 along with a negative hospital patient, low positive hospital patient and a high positive hospital patient are tested for new kit validations.

Kit Lot Entry in the LDMS system

Log in Roche kit information into the QA/QC Kit Entry module. This includes kit lot, QS input, kit controls and ranges. Separate entry is required for each assay used- e.g. Standard and UltraSensitive.

Log in VQA control information into the QA/QC Kit Entry module, including lot and ranges (VQA control information sheets may be retrieved from the website at http://aactg.s-3.com/vqa.htm). Separate entry is required for each assay used- e.g. Standard and UltraSensitive

It is required that the results be reviewed and QAed by the supervisor, director, or designee before they are released. Any suspect samples should be censored in LDMS so that they are not exported before repeat testing is performed.

Corrective Actions

8.6 The entire run is considered to be invalid if any one of the controls are not within specified limits. Repeat the entire test process: specimen and control preparation, reverse transcription, amplification, and detection.

If controls are consistently outside the specified limits, contact the Roche Response Center® for technical assistance. If one technologist is experiencing a problem with controls, but other technologists are not, then retraining might be necessary.
If one of the positive control values falls outside of the 2 standard deviations, but within 3 standard deviations, (typically with in the kit range) the run can be accepted. When the assay is run the next time and same control is out, further investigation needs to be done. If both positive controls have values outside of 2 standard deviations but within 3 standard deviations and within the kit range, the results should be reviewed by the laboratory director, supervisor or designee for final approval.

9. Troubleshooting/Maintenance

9.1 Maintenance is to be performed on the plate washer, reader, and thermal cycler The maintenance on the plate washes will vary depending on the model and brand but the system should be decontaminated with Isoporpanol **weekly. The plate reader needs to be calibrated daily along with monthly reproducibility, repeatability and turn around.** There is daily, weekly, monthly**,** and semi- annual maintenance to be performed on the thermal cycler. Check the **Applied Biosystems**/Perkin Elmer manual **and the laboratory maintenance manual for detailed instructions. Complete the appropriate maintenance sheets posted by the plate washers, plate reader and thermocyclers**

9.2 To troubleshoot any problems with the assay itself, please call Roche PCR technical support at 1-800-526-1247. Our account number is 55081604. Any problems noted with the kit should be reported to the supervisor, director, and the company.

10. Procedural Note

Interfering Substances

10.1 Interfering substances include but are not limited to the following:
Heparin inhibits PCR; specimens collected using heparin as the anticoagulant should not be used with the AMPLICOR HIV-1 MONITOR Test, v1.5.

Elevated levels of lipids, bilirubin, total protein and hemoglobin in specimens do NOT interfere with the quantitation of HIV-1 RNA by this test.

The following drug compounds do NOT interfere with the quantification of HIV-1 RNA by this test: Indinavir, ritonavir, nelfinavir, saquinavir, zidovudine, didanosine, zalcitabine, stavudine, lamivudine, HBY 097, nevirapine, delaviridine, ganciclovir, and ganciclovir valinate.

Procedural Limitations

10.2 Monitoring the effects of antiretroviral therapy by serial measurement of plasma HIV-1 RNA has only been validated for patients with baseline viral loads $\geq$ 25 000 copies/mL.

When testing specimens with viral loads <200 copies/mL, the user should consider the use of well-characterized reference materials titered from 50 copies/mL to 200 copies/mL, inclusive.

With the Standard specimen processing procedure, the test can accurately detect a 0.5 log10 (3-fold) or greater change in HIV-1 RNA if the viral load is greater than 1,000 copies/mL and a 0.78 log10 (6-fold) or greater change if the viral load is 400-1,000 copies/mL.

When compared to the AMPLICOR HIV-1 MONITOR Test, v1.0, the AMPLICOR HIV-1 MONITOR Test, v1.5 demonstrated improved efficiency for HIV-1, Group M, subtypes A-H. However, the linearity, reproducibility and limit of detection have not been evaluated for non-B subtypes.

The AMPLICOR HIV-1 MONITOR Test, v1.5, is not intended to be used as a screening test for HIV-1 or as a diagnostics test to confirm the presence of HIV-1 infection.

The AMPLICOR HIV-1 MONITOR Test, v1.5, appears to have higher variation compared to v1.0.

This test has only been validated for use only with human plasma collected in EDTA or ACD anticoagulants. Testing other specimen types may result in false negative or false positive results.
The presence of AmpErase in the Master Mix reduces the risk of amplicon contamination. However, only good laboratory practices and careful adherence to the procedures and precautions in the Method Manual may avoid contamination.
It is recommended the user re-establish the baseline viral load level for non-B subtypes.
Studies have demonstrated that the AMPLICOR HIV-1 MONITOR Test, v1.5 produces results that are comparable or enhanced from results generated from the previous version.
Only personnel with special training in PCR techniques should use this product.
Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.

Procedural Precautions

10.3 Workflow in the laboratory must proceed in a unidirectional manner. It must begin in the Reagent Preparation area, move to the Specimen Preparation area, and then move to the Amplification/Detection area.
Reagent preparation and specimen preparation are performed in separate, segregated areas. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Gloves must be worn in each area and must be removed before leaving that area.

Supplies, equipment, and gloves used for the preparation activities must not be used in the Amplification/Detection activities. Any amplification and detection supplies and equipment must remain in that area at all times.
All pipettors, pipettes, bulbs, pipette tips, etc. must be dedicated to, and used only for, its individual PCR activity. It must not be used for any non-PCR activity.

Technical Assistance from Manufacturer

For telephone technical assistance:

Roche Response Center®: 1-800-526-1247

PERFORMANCE CHARACTERISTICS:

A. Limit of Detection, Linear Range and Precision

The analytic performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 using the Standard specimen preparation procedure was determined by testing serial dilutions of a well characterized stock of cultured HIV-1 virus into HIV-negative human plasma. The virus stock, a HIV-1 group M subtype B isolate, was provided by the Virology Quality Assurance (VQA) Laboratory of the AIDS Clinical Trials Group' (ACTG), Division of AIDS, National Institutes of Health, Bethesda, MD, USA. The concentration of viral RNA in the virus stock was estimated by electron microscopy, p24 antigen concentration, the AMPLICOR HIV-1 MONITOR Test, and branched chain DNA Analysis.

These panels were tested in multi-sites with multi-operator (2 within sites) and multi-reagent lots (3). The study was carried out for 8 days per site, per lot, assaying 3 aliquots of each panel member each day.

B. Limit of Detection

Limit of detection is defined as the viral concentration at which the sample can be detected as reactive at a rate of 95% or better. The data from this study are shown in Table 2 for the Standard specimen preparation procedure.

Table 2

Limit of Detection, Standard Specimen Preparation

HIV-1 RNA (copies/mL)	Positive Results	Negative Results	Sensitivity	(95% CI)
175	98	26	0.79	0.71, 0.86
250	101	20	0.83	0.76, 0.90
400	115	7	0.94	0.89, 0.98
500	119	2	0.98	0.94, 1.00
600	121	4	0.97	0.92, 0.99
2000	113	0	1.00	0.97, 1.00
7000	125	0	1.00	0.97, 1.00
35000	123	0	1.00	0.97, 1.00
100000	124	0	1.00	0.97, 1.00
350000	121	0	1.00	0.97, 1.00
500000	126	0	1.00	0.97, 1.00
650000	119	0	1.00	0.97, 1.00
750000	126	0	1.00	0.97, 1.00
850000	122	0	1.00	0.97, 1.00

These results show that the limit of detection of the version 1.5 assay for the Standard specimen preparation procedure is 400 HIV-1 RNA copies/mL.

C. Linear Range

The linear range was determined by regression analysis using expected HIV-1 RNA concentrations and the corresponding test results.

Figure 3
Linearity of the AMPUCOR HIV-1 MONITOR Test, version 1.5
Standard Specimen Preparation Procedure

As shown in Figure 3, the AMPLICOR HIV-1 MONITOR Test, version 1.5 with Standard specimen processing was found to give a linear response from 400 (log10 = 2.60) to at least 750,000 (log10 = 5.85) HIV-1 RNA copies/mL Patients with viral load results less than 100,000 HIV-1 RNA copies/mL should be monitored using the UltraSensitive specimen preparation procedure.

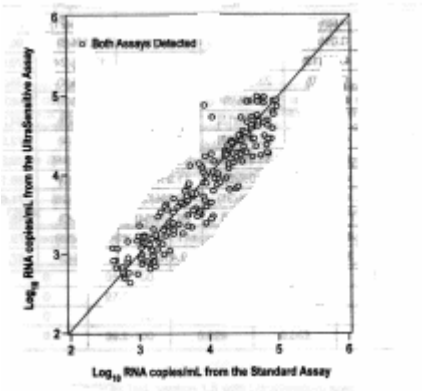
CORRELATION OF RESULTS FOR STANDARD AND ULTRASENSITIVE SPECIMEN PREPARATION PROCEDURES

In studies conducted at three separate laboratories, 181 patient specimens were tested by the AMPLICOR HIV-1 MONITOR Test, version 1.5 using both the Standard and UltraSensitive specimen preparation procedures. The overall correlation for results combined across laboratories was 0.932 with a bias towards lower values (0.0029 Log10 lower by the UltraSensitive procedure).

Figure 5

AMPL/COR HIV-1 MONITOR Test, version 1.5 Correlation of Standard and

UitraSensitive Specimen Preparation Procedures



D. Precision

For the Standard specimen preparation procedure of the AMPLICOR HIV-1 MONITOR Test, version 1.5, Coefficients of Variation (CV's) in the linear range were between 30% and 94%, while the prior version of the assay had CV's of between 32.3% and 45.3%.

Table 4

Precision of the AMPL/COR HIV-1 MONITOR Test, version 1.5, Components of Variance Analysis; Summary of Performance Results from S1andard Specimen Preparation Procedure

Input HIV-1 RNA (copies/mL)	Total Standard Deviation (copies/mL)	Attribution Percentage of Total Variance (%CV)					
		Kit lot	Study Center	Operator	Day to Day	Within Run	Total CV
175	236	0.0% (0)	0.0% (0)	1.2% (8)	11.2% (25)	87.6% (69)	74%
250	163	1.64% (7)	0.0% (0)	0.% (0)	14.6% (22)	83.9% (52)	56%
400	277	0.0% (0)	0.0% (0)	2.0% (8)	9.7% (17)	88.3% (50)	54%
500	389	0.0% (0)	0.0% (0)	6.9% (16)	6.9% (16)	86.3%(57)	61%
600	380	0.0% (0)	0.0% (0)	3.8% (10)	10.4% (17)	85.8% (48)	52%
2000	826	0.0% (0)	4.0% (7)	5.1% (8)	0.0% (0)	90.9% (34)	36%
7000	2294	0.0% (0)	10.8% (10)	0.0% (0)	9.2% (9)	80.1% (27)	30%
35000	14841	4.7% (8)	6.3% (10)	10.5% (12)	5.9% (9)	72.6% (33)	38%
100000	40791	0.7% (8)	4.3% (7)	4.6% (8)	4.9% (9)	86.2% (36)	39%
350000	372502	0.0% (0)	8.1% (27)	23.4% (46)	33.5% (55)	35.0% (56)	94%
500000	328155	0.4% (4)	25.2% (33)	1.9% (9)	15.7% (26)	56.8% (50)	66%
650000	497804	0.0% (0)	21.5% (37)	9.3% (24)	6.7% (20)	62.5% (62)	79%
750000	481108	0.0% (0)	17.1% (29)	11.2% (24)	0.0% (0)	71.7% (60)	71%
850000	356316	0.0% (0)	26.5% (25)	21.6% (23)	0.0% (0)	51.9% (35)	49%

E. Analytical Specificity

The analytical specificity of the AMPLICOR HIV-1 MONITOR Test, version 1.5 was evaluated by adding cultured cells, cultured virus or purified nucleic acid from the following organisms and viruses into HIV negative human plasma (after Step 81.6 of the *"Instructions for* Use"), then analyzing these samples. None of the non-HIV organisms, viruses or purified nucleic acids tested showed reactivity in the AMPLICOR HIV-1 MONITOR Test, version 1.5 when performing the Standard specimen preparation procedure. Two of the four HIV-2 isolates tested (7824A and 60415K) yielded positive results; however, no specific claims can be made for the ability of this test to amplify HIV-2 isolates (Table 6).

Table 6
List of Organisms, Cultured Virus, or Purified Nucleic Acid Tested with the AMPLICOR HIV-1 MONITOR Test, version 1.5

Adenovirus type 2	Hepatitis C Virus Genotype 4c	Human Herpes simplex type I, strain Macintyre
Adenovirus type 3	HIV-2, subtype A/B* (Isolate 7312A)	Human papilloma virus 6b
Adenovirus type 7	HIV-2, subtype A* (Isolate 7824A)	Human papilloma virus 11
Candida albicans	HIV-2, BEN*	Human papilloma virus 16
Cytomegalovirus AD-169	HIV-2, subtype A* (ISOlate 6O415K)	Human papilloma virus 18
Cytomegalovirus Davis	HTLV-1, C5/MJ cells	*Mycobacterium avium*
Cytomegalovirus Towne	hTLV-1, 0S-P2 cells	*Pneumocystis carinii*
Epstein-Barr virus (RAJI- Burkitt's Lymphoma cells)	HTLV-I, MT-2 cells	*Propionibacterium acnes*
Epstein-Ban virus HR1 (Burkitt's Lymphoma cells)	HTLV-I, MJ cells	*Staphylococcus aureus*
Epstein-Ban virus P-3 (Burkitt's Lymphoma cells)	HTLV-II, MoT cells	*Staphylococcus epidermidis*
Hepatitis B Virus *(full length clone)*	HTLV-II (full length clone)	*Varicella-Zoster Ellen*
Hepatitis B Virus (patient sera)	Human Herpes Virus 6	*Varicella Oka*
Hepatitis C Virus Genotype 1 a	Human Herpes Virus 7	
Hepatitis C Virus Genotype 1b	Herpes simplex type I, strain F	
Hepatitis C Virus Genotype 2a/2c	Human Herpes simplex type I, strain HF	
Hepatitis C Virus Genotype 2b	Human Herpes simplex type II, Strain MS	
Hepatitis C Virus Genotype 3a	Human Herpes simplex type II, strain G	

*2 of 4 HIV-2 isolates yielded positive results.

CLINICAL SPECIFICITY

Standard Specimen Preparation

The clinical specificity of the AMPLICOR HIV-1 MONITOR Test, version 1.5 with Standard specimen preparation was determined by analysis of 459 specimens from anti-HIV-1 negative blood donors. Specimens containing either EDTA or ACD as anticoagulant were tested with three lots of the AMPLICOR HIV-1 MONITOR Test, version 1.5. None of these specimens were reactive with the AMPLICOR HIV-1 MONITOR Test, version 1.5 (Table 7). Assuming a zero prevalence of HIV-1 infection in the seronegative blood donors, the specificity of the test was 100% [95% CI = (99.2-100%)].

Table 7
Anti-HIV-1 Negative Blood Donors Specificity Analysis of *Serology Negative Donor specimens,*
Standard Specimen Preparation Procedure

HIV-1 RNA				Distribution of Abosrbency (A_{450}) Values				
Kit Lot #	Not Detected	Detected	95% CI	Minimum	Average	Maximum	SD	%CV
1	84	0	95.7-100	0.048	0.062	0.093	0.010	17
2	146	0	97.5-100	0.047	0.067	0.132	0.017	26
3	229	0	98.4-100	0.029	0.059	0.160	0.017	28
Overall	459	0	99.2-100	0.029	0.062	0.160	0.016	26

INCLUSIVITY: PERFORMANCE OF HIV-1 GROUP M Non-B Subtypes

The primary purpose for developing the AMPLICOR HIV-1 MONITOR Test, version 1.5 was to improve the detection efficiency of Group M non-B subtypes as compared to the version 1.0 assay. Three studies were conducted to evaluate the performance of the version 1.5 on HIV-1 group M subtypes A to H.

Study 1

Study material for 14 of 16 isolates used in this study came from 30 HIV-1 isolates of known HIV-1 Group M subtypes that were cultured and characterized by electron microscopic particle counting, infectious titer, p24 Ag concentration, and reverse transcriptase activities. These virus stocks were prepared in collaboration with Walter Reed Army Institute of Research. The remaining two isolates are part of a panel of 44 HIV-1 isolates of known subtype that were cultured and characterized by electron microscopic particle counting as part of an effort by AIDS Clinical Trial Group (ACTG) Virology Quality Assurance Program to produce a set of international HIV-1 standards. This panel includes all 30 isolates in the Roche-Walter Reed panel.

Sixteen panels were prepared from different HIV-1 isolates of subtypes A, B, C, D, E, F, G and H. Each panel consisted of 7 concentrations. The study was carried out at three sites. Each site received

the panels with blinded numbers and each panel (n=7 Standard and 7 UltraSensitive Specimen Preparation panel members) was tested using 3 manufacturing lots of reagents.

The mean results for all concentration levels of all panel members at each dilution were used to compare the performance of version 1.5 to that of version 1.0 for both sample preparation methods. Figures 8 and 9 present the mean test results for the AMPLICOR HIV-1 MONITOR Test, version 1.5 and 1.0, respectively, when the Standard specimen preparation procedure was used. Examination of these results indicate that, for both specimen preparation procedures, with all Group M, non-B isolates, the AMPLICOR HIV-1 MONITOR Test, version 1.5 demonstrates improved performance compared to version 1.0 of the assay.

These results show that the AMPLICOR HIV-1 MONITOR Test, version 1.5 provides comparable or enhanced performance in amplification of specimens containing HIV-1 Group M, subtype A-H when compared with version 1.0 of the same assay.

Figure 8
Performance of AMPL/COR HIV-1 MONITOR Test, *version* 1.5 *on HIV-1 Subtypes* A *through H, when using the Standard Specimen Preparation Procedure*

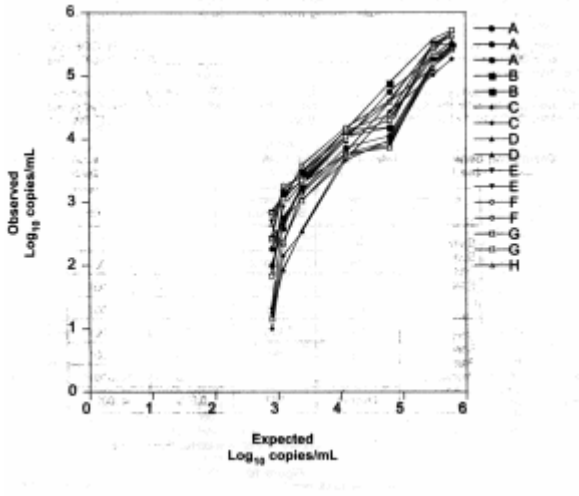
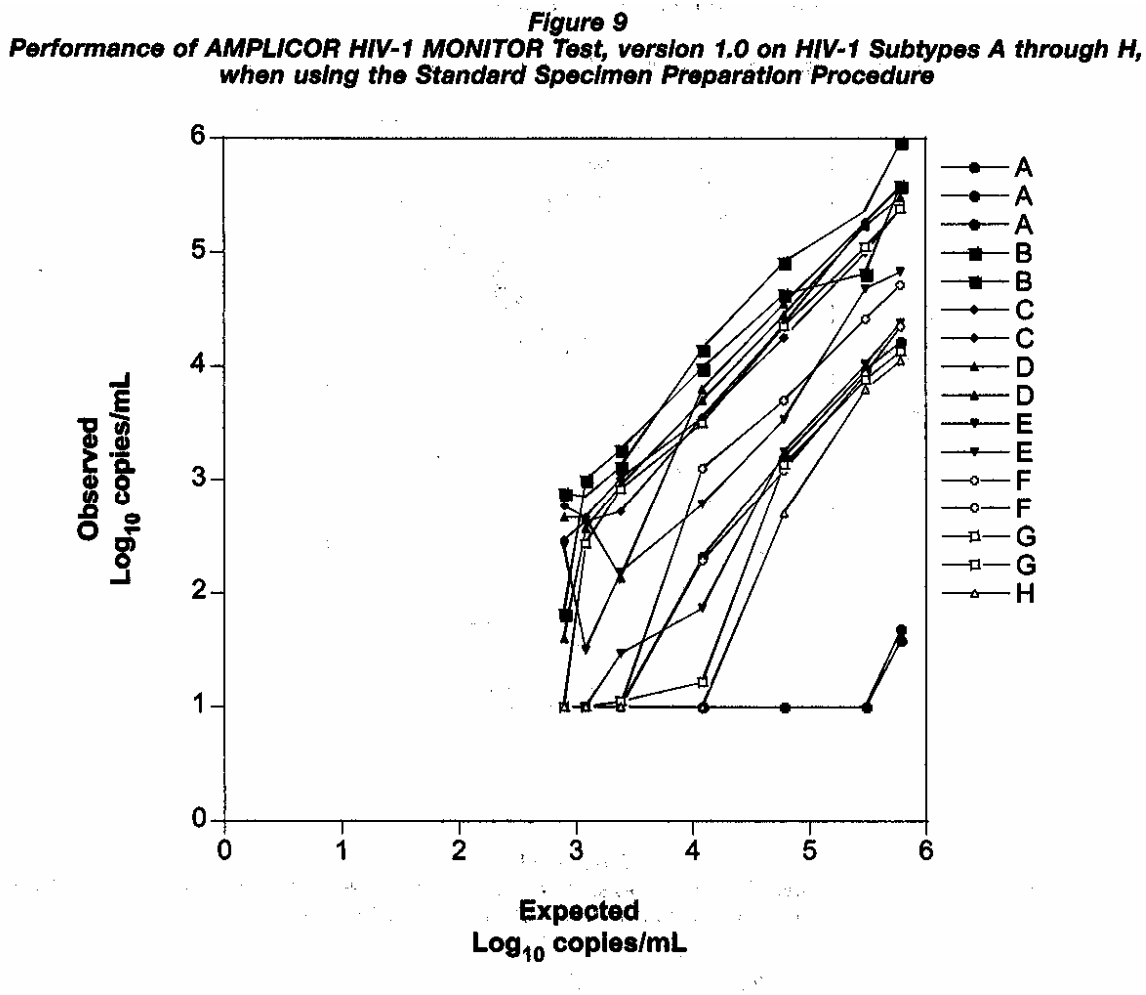


Figure 9 Performance of AMPL/COR HIV-1 MONITOR Test, version 1.0 on HIV-1 Subtypes A through H, when using the Standard Specimen Preparation Procedure



Study 2

The performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 with the specimen processing procedure was further evaluated by assaying the World Wide HIV-1 subtype performance panel (WWRB301) from Boston Biomedica, Inc. The panel consists of fifty, plasma specimens from HIV-1 infected patients from Africa, Asia, South America and North America. This well characterized panel contained data on the subtype of each HIV-1 infection, viral load results (Chiron Quantiplex, Organon Teknika NASBA, AMPLICOR HIV-1 MONITOR Test, version *1.0),* and serology test results.

In this study three trial sites participated, analyzing all 50 members of the panel using both sample preparation methods.

Examination of these data suggests that the AMPLICOR HIV-1 MONITOR, version 1.5 assay, with the Standard specimen preparation procedures provide consistently better results compared to the AMPLICOR HIV-1 MONITOR, version *1.0,* assay, Study 3

This study was conducted in a clinical setting in the United Kingdom, where patients with HIV-1 Group M, non-B subtype infections were monitored using the AMPLICOR HIV-1 MONITOR Test, version 1.5, The data was collected as part of a clinical audit. Patient inclusion criteria included: 1) having infection with HIV-1 group M non-B subtype, as defined by serologic testing and partial sequences of the HIV-1 genome, and 2) having been tested at 5 or more time points using the AMPLICOR HIV-1 MONITOR Test, version 1.5 during a period when antiretroviral drug therapy was initiated or switched. Twenty eight (28) of 152 patients met the criteria for inclusion, This subpopulation was composed of 16 males and 12 females. Twenty-six of the patients received a 3 drug (or more) therapy. Upon initiation or switching of drug therapy, 16 of these patients had at least one viral concentration below the limit of detection while the remaining 12 patient's viral load dropped to an average of 19,800 copies/mL during the study. All those patients who did not achieve undetectable viral loads in response to therapy were noted to have adherence problems or were on suboptimal therapy, These results indicate the AMPLICOR HIV-1 MONITOR Test, version 1.5 was useful in monitoring patients infected with HIV-1 Group M non-B subtypes for antiretroviral drug therapy.

Performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5, Compared to the AMPLICOR HIV-1 MONITOR Test, version 1.0

The performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 was compared to that of the AMPLICOR HIV-1 MONITOR Test, version 1.0 by testing archived HIV-1 reactive plasma samples with prior AMPLICOR HIV-1 MONITOR, version 1.0 test results. All samples were collected in the United States and were presumed to be subtype B. Three hundred seventy one (371) such samples with AMPLICOR HIV-1 MONITOR Test, version 1.0 Standard specimen preparation procedure test results ranging from 400 to 750,000 HIV-1 RNA copies/mL were tested using the AMPLICOR HIV-1 MONITOR Test, version 1.5 Standard specimen preparation procedure. The data are presented in Figure 10.

As summarized in Table 9, the test results obtained with the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 are highly correlated for both the Standard specimen preparation procedure and UltraSensitive specimen preparation procedure. Thus, the test results of the AMPLICOR HIV-1 MONITOR Test, version 1.0 and the AMPLICOR HIV-1 MONITOR Test, version 1.5 were comparable.

Table 9

Correlation between the AMPLICOR HIV-1 MONITOR Tes1 Assays, versions 1.0 and 1.5 with the Standard and UltraSensitive Specimen Preparation Procedures

Method	Site	# of specimens	Correlation	P Value
Standard Specimen Preparation Procedure	04	113	0.942	<.0001
	05	113	0.972	<.0001
	06	145	0.965	<.0001
	Overall	371	0.961	<.0001
UltraSensitive Specimen Preparation Procedure	04	116	0.980	<.0001
	05	98	0.976	<.0001
	06	133	0.976	<.0001
	Overall	347	0.976	<.0001

1 *Correlation by Spearman's coefficient of rank correlation method.*

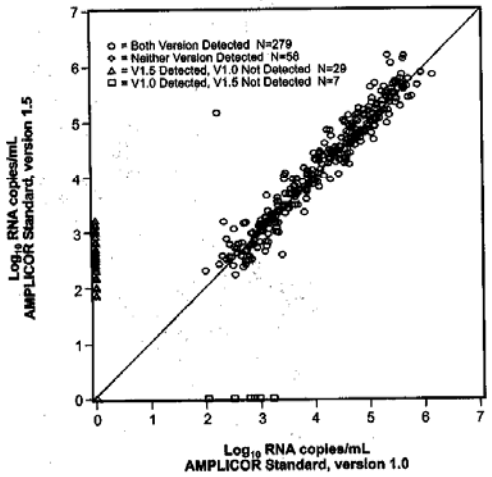
Figure 10

Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Plasma Specimens from HIV-1 Infected Patients

Standard Specimen Preparation Procedure

Figure 10
Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Plasma Specimens from HIV-1 Infected Patients

Standard Specimen Preparation Procedure



CLINICAL PERFORMANCE

The clinical utility of the AMPLICOR HIV-1 MONITOR Test was demonstrated using the version 1.0 test only. Since the analytical performance for the AMPLICOR HIV-1 MONITOR Test, version 1.5, is similar to or better than that of version 1.0, the data presented here for version 1.0 are expected to be applicable to version 1.5.

Patient Prognosis

The use of the AMPLICOR HIV-1 MONITOR Test to predict the risk of disease progression in HIV infected individuals was evaluated in ACTG studies 116A and 116B/117. The data from these studies were analyzed by the Cox Proportional Hazards Model to evaluate the frequency of disease progression based upon HIV-1 RNA level. ACTG Study 116A was a double-blinded study that compared the clinical efficacy of zidovudine (ZDV) in combination with two doses of 2',3'dideoxyinosine (ddl) in patients with advanced HIV disease who had up to 16 weeks of prior treatment with zidovudine. ACTG Study 116B/117 was an efficacy study which compared 2',3'dideoxyinosine (ddl) and zidovudine therapy of patients with HIV infection who had been on zidovudine treatment for more than 16 weeks. The patient population in each of these studies included

patients with a diagnosis of AIDS at study baseline, ARC at baseline and asymptomatic patients at baseline. Disease progression was defined as progression to AIDS, a new AIDS defining event, or death.

The ACTG 116A HIV-1 RNA sub-study included 186 patients from the clinical study who had had plasma and PBMC specimens collected upon entrance into the study and whose specimens had been stored appropriately and were available for testing. The ACTG 116B/117 HIV-1 RNA sub-study included 99 randomly selected patients from the drug study who had plasma specimens collected on entrance into the study and whose specimens had been stored appropriately and were available for testing.

The unadjusted and adjusted relative hazards for disease progression as measured by baseline HIV-1 RNA levels, change in HIV-1 RNA levels over 8 weeks, and CD4+ cell counts were evaluated using Cox Proportional Hazards Models. The unadjusted Relative Hazard represents the risk conferred by the variable alone, whereas the Adjusted Relative Hazard represents the risk conferred by that variable after controlling for the other variables in the model. These models give the increased risk (if any) of disease progression associated with the variables entered into the model. These analyses were performed by assessing the relative hazards of a 5-fold difference in the study variable. The results of these analyses are presented in Tables 10 through 14. These data show that in a population of patients with advanced HIV disease and undergoing specific anti-reverse transcriptase therapies, 5-fold higher baseline HIV-1 RNA levels are associated with increased risk of disease progression. For patients who have had greater than 16 weeks of prior ZDV therapy (patients in ACTG Study 116B/117), 5-fold higher baseline HIV-1 RNA levels were not of statistically significant prognostic value. For patients who have had either no prior ZDV therapy or 16 weeks or less of ZDV therapy, 5-fold changes between baseline and Week 8 RNA levels have statistically significant prognostic value. For patients who have had greater than 16 weeks of prior ZDV therapy, 5-fold changes between baseline and Week 8 RNA levels have not proven to be of significant prognostic value. The frequency of disease progression was also analyzed for each study by dividing each study population into deciles by rank order of base- line HIV-1 RNA. The deciles were evaluated for the frequency of disease progression. For each study, a frequency of disease progression of 60% was found for all patients with baseline HIV-1 RNA levels above 250,000 copies/mL. In Study 116A, an approximate 35% frequency of disease progression was found for patients in the first four deciles « 11912, < 34,661, < 72,438 and < 1 03,806 HIV-1 RNA copies/mL). The frequency of disease progression was between 40% and 50% in the nex1 three deciles « 150695, < 194,312 and < 247,229). In the last three deciles, where the HIV-1 RNA levels were> 250,000 HIV-1 RNA copies/mL, the frequency of disease progression was greater than 60%. In Study 116B/117 the frequency of disease progression was found to be more variable, but still showed the general trend of higher rates of progression with increased HIV-1 RNA levels. An average 30% disease progression rate (range = 10% -60%) was found for the first six deciles in this study (<11,571, < 31,292, < 49,743, < 62132, < 97,781 and < 150,866). The rate of disease progression increased to 50% and 60% for the nex1two deciles (<251,627 and < 403,146, respectively). The disease progression rates were greater than 60% for the last two deciles (< 794,027 and < 1 ,456,302) where the HIV-1 RNA levels were greater than 4O3,000. Summary tables and bar charts of these analyses are presented in Figures 12 and 13.

Table 10

Association of Study Variables at Baseline with Disease Progression ACTG Study 116A (N = 153 Patients, 73 Progression Events)

Variable	Unadjusted Relative Hazard (95% CI)		Adjusted Relative Hazard (95% CI)	p value2	
Log HIV-1 RNA copy number	1.58 (1.20 -2.09)		1.44 (1.07 -1.93)	0.02	
Log CD4+ cell count	0.39 (0.28 -0.54)		0.45 (0.31 -0.64)	0.0001	Dx of AIDS at Baseline 2.00 (1.22 -3.27)
		1.39 (0.82 -2.37)	0.22		
ddl Treatment	0.95 (0.59 -1.53)		1.12 (0.68 -1.64)	0.66	

1 -The Relative Hazard is the hazard ratio resulting *from* a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2 -p values are for Adjusted Relative Hazards

Table 11

Association of Change in HIM RNA from Baseline to Week 8 with Disease Progression ACTG Study 116A (N = 114 Patients, 62 Progression Events)

Variable	Unadjusted Relative Hazard (95% CI)	Adjusted Relative Hazard (95% CI)	p value2
LogHIV-1RNAcopy number1	1.46(1.11-1.93)	1.63(1.16-2.28)	0.0005
LogChangeinHIV-1RNA *from* Baseline to Week 81	1.18(0.93-1.48)	1.54(1.09-2.16)	0.013
Log CD4+ cell count1	0.43 (0.30 -0.62)	0.50 (0.34 -0.73)	0.0004
Dx of AIDS at Baseline	1.83 (1.09 -3.09)	1.28 (0.74 -2.21)	0.38
ddl Treatment	0.76 (0.45 -1.27)	0.87 (0.51 -1.49)	0.61

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2 -p values are for Adjusted Relative Hazards ~

Table 12
A*ssociation of Study Variables at Baseline with Disease Progression ACTG Study 116B/117 (N =* 86 *Patients,* 39 *Progression Events)*

Variable	Unadjusted Relative Hazard (95%CI)	Adjusted Relative Hazard (95% CI)	p value[2]
Log HIV-1 RNA copy number1	1.90 (1.28 -2.82)	1.25 (0.81 -1.94)	0.32
Log CD4+ cell count1	0.28 (0.16 -0.48)	0.33 (0.18 -0.62)	0.0006
Dx of AIDS at Baseline	3.13(1.66-5.92)	2.38(1.24-4.58)	0.01
ddl Treatment	0.89 (0.47 -1.69)	0.88 (0.46 -1.71)	0.71

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2'- P values are for Adjusted Relative Hazards

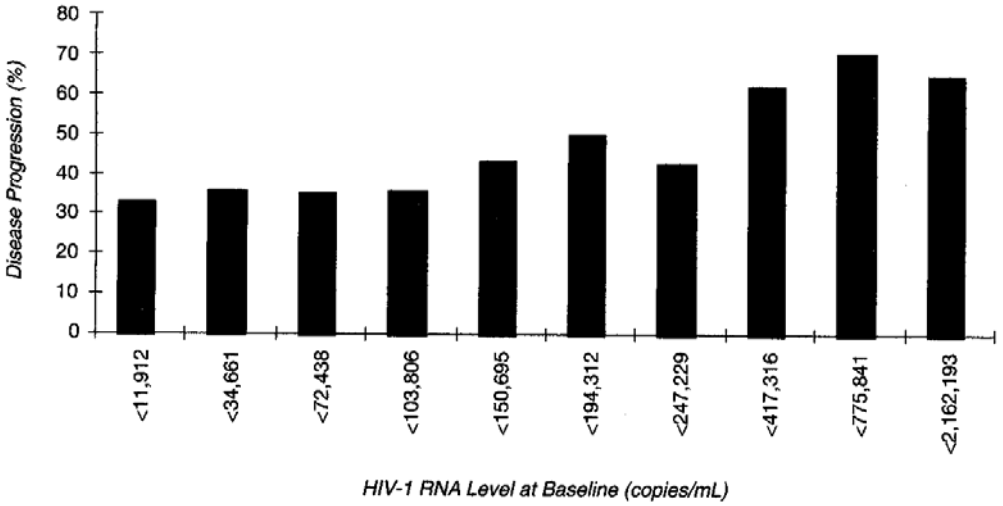
Table 13
Association of Change in HIM RNA from Baseline to Week 8 *with Disease Progression ACTG Study 116B/117 (N =* 65 *Patients,* 29 *Progression Events)*

Variable	Unadjusted Relative Hazard (95%CI)	Adjusted Relative Hazard (95% CI)	p value[2]
LogHIV-1RNAcopy number1	2.10(1.32-3.34)	1.58(0.93-2.69)	0.09
Log Change in HIV-1 RNA from Baseline to Week 81	1.41 (0.71 -2.79)	1.58 (0.68 -3.68)	0.29
Log CD4+ cell count1	0.25 (0.13 -0.46)	0.29 (0.14 -0.60)	0.001
Dx of AIDS at Baseline	2.43(1.17-5.05)	1.87(0.88-3.97)	0.10
ddl Treatment	1.07 (0.51 -2.27)	0.98 (0.39 -2.48)	0.96

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

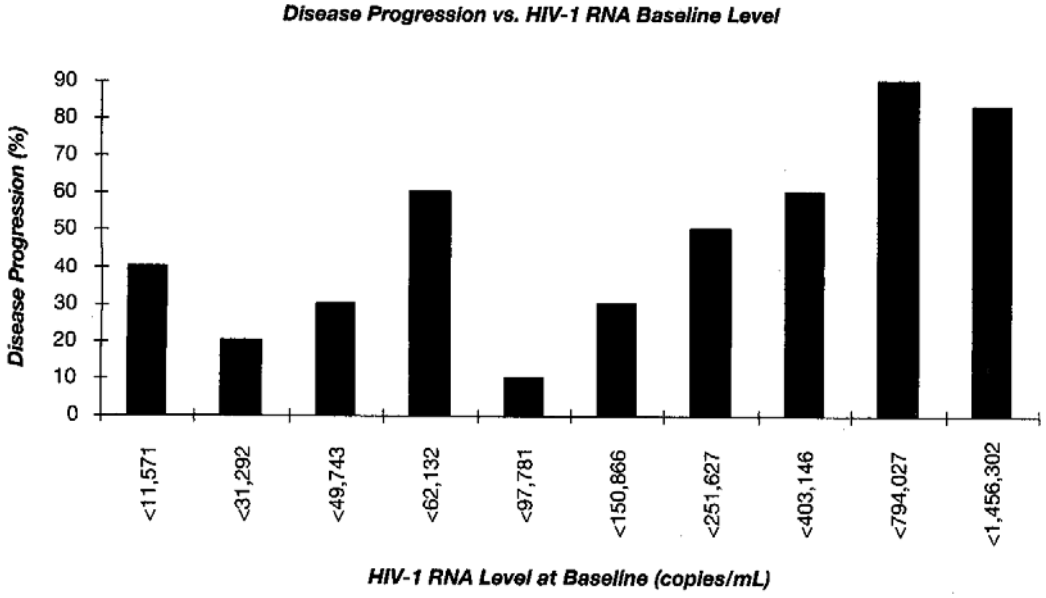
2 -P values are for Adjusted Relative Hazards

Figure 12
Frequency of Disease Progression by Baseline HIV-1 RNA Level
Study 116A



Record #	Decile	n=	# Progressions	# Progressions to AIDS/Death	%
18	<11912	18	15	5	33.33
36	<34661	18	14	5	35.71
54	<72438	18	17	6	35.29
72	<103806	18	14	5	35.71
90	<150695	18	16	7	43.75
108	<194312	18	16	8	50.00
126	<247229	18	14	6	42.86
144	<417316	18	16	10	62.50
162	<775841	18	17	12	70.59
179	<2162193	17	14	9	64.29

Figure 13
Frequency of Disease Progression by Baseline HIV-1 RNA Level
Study 116B/117



Record #	Decile	n=	# Progressions	# Progressions to AIDS/Death	%
10	<11571	10	10	4	40.00
20	<31292	10	10	2	20.00
30	<49743	10	10	3	30.00
40	<62132	10	10	6	60.00
50	<97781	10	10	1	10.00
60	<150866	10	10	3	30.00
70	<251627	10	10	5	50.00
80	<4031	46	10	10 6	60.00
90	<794027	10	10	9	90.00
96	<456302	6	6	5	83.33

Measuring Response to Antiretroviral Therapy

The use of the AMPLICOR HIV-1 MONITOR Test to measure the effects of antiretroviral therapy was evaluated in a clinical study of antiretroviral compounds including the reverse transcriptase inhibitor zalcitabine (ddC, tradename HIVID), the protease inhibitor drug Saquinavir (SAO, tradename INVIRASE, and combinations of these two drugs. The study (Study NV14256) was a double-blind, phase 111 randomized study whose primary objective was to evaluate the safety, tolerability and efficacy of three treatments (ddC, SAO, and SAO in combination with ddC) based on clinical endpoints in patients discontinuing (or unable to take) zidovudine (ZDV) therapy and to compare survival among the three treatment groups [including death after an AIDS Defining Event (ADE), or dose limiting toxicity].

For Study NV14256, prior ZDV treatment was an inclusion criterion with the majority of patients having over one year of prior ZDV treatment, A total of 970 patients were entered into Study NV14256 at forty nine clinical sites. The study had the following three treatment arms (patients): ddC (325), Saquinavir (327), and Saquinavir + ddC (318), The demographic and baseline disease characteristics of the patients in Study NV14256 represented a diverse population of patients with advanced HIV-1 infec1ion and with a wide range of prior antiretroviral treatment regimes.

The utility of the AMPLICOR HIV-1 MONITOR Test to measure the effects of drug treatment over time was evaluated by analyzing the median change from baseline and the DAVGt (difference

averaged over time -mean change from baseline over t weeks) in the HIV-1 RNA level, To evaluate the ability of the AMPLICOR HIV-1 MONITOR Test to detect changes in HIV-1 RNA levels as a result of therapy, the median change from baseline was analyzed over time for each treatment arm of the study, Figure 14 shows the median change from baseline over a 48 week time period for each treatment arm in Study NV14256. Measurable and sustained decreases in HIV-1 RNA levels as determined by the AMPLICOR HIV-1 MONITOR Test were seen. The largest and the most sustained median change in HIV-1 RNA levels at each time point was seen in patients in the combination treatment arm (SAO+ddC).

The utility of serial HIV-1 RNA measurements to assess viral response to antiretroviral treatment was also examined using the Cox Proportional Hazards Model, In this analysis, the three treatment groups were combined and a Cox model, stratified by treatment group, was fit using the following covariates as linear terms: 10910 (baseline HIV-1 RNA), 10910 (last HIV-1 RNA), baseline CD4+ and last CD4+. The hazard ratio in this model was defined as a 10-fold increase in HIV-1 RNA or a decrease of 100 in CD4+ count. As shown in Table 14, the coefficient of the log10 (last HIV-1 RNA) term for this model is statistically significant and positive through Week 40, Accordingly, the HIV-1 RNA Hazard Ratios through Week 40 indicate that the HIV-1 RNA levels et each time point have statistically significant and continuous prognostic value.

In a related analysis of the data, the association between HIV-1 RNA levels and survival time was assessed. For this analysis, survival time was defined as time on study in which a patient survived free of an AIDS Defining Event (ADE) or death. The analyses were performed by constructing Kaplan-Meier Survival Curves for each treatment arm by dividing the patient population within the treatment arm into thirds based on HIV-1 RNA levels, nominally the Lower, Middle, and Upper thirds. The survival curves were plotted as a function of the percent ADE Survival for each patient group (Lower, Middle and Upper) for the number of weeks through Week 8. The Kaplan Meier estimated survival analyses show that patients with low HIV-1 RNA levels (Lower Third) are more likely to survive ADE-free for a longer period of time than patients with high HIV-1 RNA levels, The Kaplan-Meier survival curves for each study treatment arm are shown in Figures 15,16 and 17.

In a separate analysis of the data from Study NV14256, a patient's risk of ADE or death was assessed as a function of the last (most recent) HIV-1 RNA level at various study weeks. In particular, the impact of the last HIV-1 RNA measurement on the hazard for the development of a first ADE or death (based upon clinical events seen later in the study) was assessed. The analysis was performed using a Cox model survival analysis using the covariates IOg10 (baseline HIV-1 RNA), IOg10 (last HIV-1 RNA), baseline CD4+ and last CD4+, The model was a linear function of the covariates predicting the log of the hazard (ratio) for an ADE. The linear function was then used to determine a Risk Index for all patients in the study. Patients who survived ADE free through Week 4 of the study were sorted by their Risk Index with the first 25% of the patients forming the "low risk" group and the remaining patients equally divided into six separate groups according to their Risk Index. Each of these six groups was then compared to the low risk group using a survival model and the mean HIV-1 RNA level of each group was plotted versus an ADE hazard ratio calculated using a piecewise exponential model. Figures 18 and 19 show the Hazard Ratios for the time to first ADE versus the 10910 (last HIV-1 RNA) for patients surviving ADE free through Week 4 and patients surviving ADE free through Week 16, respectively. These data show that a patient in this study at Week 4 with an HIV-1 RNA level of 100,000 copies/mL (10910 = 5.0) was 6 times more likely to develop an ADE or to die than was patient with an HIV-1 RNA level of 8000 copies/mL. A patient in this study at Week 16 with

an HIV-1 RNA level of 1,000,000 (log10 = 6.0) was 20 times more likely to develop an ADE or to die than was a patient with an HIV-1 RNA level of 8,000 copies/mL.
The data presented here regarding the clinical utility and the use of the AMPLICOR HIV-1 MONITOR Test to monitor the effects of antiretroviral therapy were derived from a single clinical study that compared two drugs and three therapeutic regimens for a specific patient group, Because a single clinical study may not adequately demonstrate the clinical utility of quantitative HIV-1 RNA testing in all patient populations, in all clinical situations, or with all antiretroviral therapies, sufficient care should be taken before extending the interpretation of the data from the study presented here to any individual patient case. As with any diagnostic test, results from the AMPLICOR HIV-1 MONITOR Test should be interpreted with consideration of all relevant clinical and laboratory findings for each patient.

Note: Some Graphs removed because of corrupted objects

10. References

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Criteria for Laboratory Approval for HIV RNA and DNA Viral Load Testing

DNA: labs will need to pass a 30-member panel before running whole blood panels. They will then need to run and pass (C) two whole blood panels to be fast-track approved for DNA testing. If they receive a PC or P on the first two panels, they will need to complete at least 4 panels and satisfy the criteria for an "approved" lab before protocol testing. The six month interval testing will occur once labs have achieved "approval". if the certification panel proceeds whole blood testing by greater than 1 year, then they'll need to recertify with a new 30-member panel.

2. RNA: labs will need to pass a 20-member panel before running real-time panels. they may begin protocol testing with the first five member panel, but the approval is "probationary" and close scrutiny of their performance during this time should occur. if a site obtains two C's on the first two panels, they are "off" probation, but if they receive a PC or P, then they remain on probation for four months at which time, depending on their performance, their ability to test protocol samples may be rescinded. we don't enforce this, the program office would, but we do make the recommendation. If the certification panel preceeds real-time testing by 6-12 months, we recommend that a site complete at least one 5 member panel (and receive a C) before beginning protocol testing. If there are problems, then more testing may be needed. If certification preceeds testing by >12 months, the lab will need to recertify with a new 20-member panel.

HIV 1 DRUG RESISTANCE ASSAY		
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SCOPE:

The genome of the Human Immunodeficiency Virus (HIV-1) undergoes mutations in an infected patient, especially in response to anti-viral drug treatment. Some of the mutations have been shown to render the virus resistant to the action of these drugs, diminishing the effectiveness of the treatment.

The detection of mutations in HIV-1 that correlate with drug resistance provides useful information to physicians in monitoring the course of treatment and selecting the most effective regimen for a particular patient.

1. PRINCIPLE

HIV genotyping by DNA sequencing is based on a modification of Sanger and colleagues dideoxynucleotide chain termination chemistry. It consists of an RT reaction that converts a specific region of the viral RNA into complementary DNA (cDNA). This specific region of HIV encompasses the genes coding for the 2 main targets of drug treatment, the protease and RT enzymes. This initial cDNA product is amplified using primers specific for protease & Reverse transcriprase genes using several nested PCRs, and the resulting amplicons are then subjected to direct sequencing. Using the ABI prism

The steps involved in HIV 1 Drug resistance assay are

1.**Viral RNA extraction**, 2**. Reverse transcription (RNA to cDNA),** 3.**Amplication using protease & RT specific primers, and 4. Purification of amplified products. , 5.Sequencing**.

2. SPECIMEN COLLECTION:

2.1.Specimen Type: Plasma
2.2.Specimen storage: Plasma specimens may be stored at:

- Room temperature for upto one day
- or Frozen at -80ºC
- Plasma specimens may be frozen & thawed upto 2 times

3.MATERIALS REQUIRED:

- **Pre-Amplification Area-Reagent Preparation**
- .Pipettors – 0.5 - 10µL ,50µL ,100µL& 1ml with aerosol barrier tips.
- Autoclaved 0.5 eppendoff tubes, 1ml eppendoff tubes & SinglePCR reaction tubes .
- Two coolers
- Reagents for master mix(MMX) preparation(Refer MMX preparation)
- Permanent markers
- Labels
- Discarding Jar

- **Pre-Amplification Area-Specimen and Control Preparation**
- 2.0 mL screw-cap tubes, sterile
- Viral RNA extraction KIT (Qiagen)
- Tube racks
- Absolute alcohol
- Pipettes 50µL, 200µL, 1000µL with aerosol barrier tips.
- Microcentrifuge (max RCF 16,000 X g, min RCF 12,500 X g)
- Refrigerated ultracentrifuge with an RCF of 23,600 x g
- Vortex mixer
- Disposable Latex gloves, powderless
- Discarding Bag & Discarding Jar (with hypo1:10)
- Permanent markers
- Autoclaved 1ml eppendoff tubes.
- **Post-Amplification Area- Amplification**
- Gene Amp 9700 Thermocycler
- Cooler (for loading samples)
- Disposable Latex gloves, powderless
- **Post-Amplification Area Detection**

- Agarose
- Electronic balance
- 50X TAE Buffer
- Conical Flask
- Butter Paper
- Gel casting tray
- Electrophoresis unit
- UV Illuminator
- Disposable Latex gloves, powderless
- Paraffin Roll
- Pipettors – 0.5 - 10µL ,50µL with aerosol barrier tips
- Loading Dye
- DNA ladder
- Microcentrifuge (max RCF 16,000 X g, min RCF 12,500 X g)
- Vortex mixer
- Discarding Jar
- Microwave(gel melting)
- ETBR(10mg/ml)

4. PROCEDURE

The HIV-1 Drug resistance test can be completed in 2 days till the amplification Stage. The products are then packed & shipped out to Macrogen Sequencing services (Korea) for purification & DNA sequencing.The turnaorund time between shippment & receiving the results from macrogen through email is normally 10 days.

4.1.Viral RNA Extraction

Kit Used - QIA amp viral RNA mini Kit

Step1- Viral concentration:

- Spin patient Plasma smples once at 2000 rpm for 10 min.
- Transfer 1 ml to 2 ml of cell free supernatant to labelled 2ml screw-cap tube for each patient specimen .
- centrifuge at 25000xg for 1hr at 4ºC
- Remove supernantant & leave about 140µL- 200 µL of plasma in the tube
- Proceed to Viral extraction.

Step 2- Viral Extraction

- Equilibrate Samples to room Temperature.

- Equilibrate Buffer AVE to room Temperature (15-25 c)
- Check that Buffer AW1, Buffer AW2,and Carrier RNA have been prepared according to the room temperature before use.
- Redissolve precipitate in Buffer AVL/ Carrier RNA by heating, if necessary, and cool to room temperature before use.
- All centrifugation steps are carried out at room temperature.

1. Pipet 560µl of prepared Buffer AVL containing Carrier RNA into a 1.5 ml micro centrifuge tube**.(***if the sample volume is larger than 140µl, increase the amount of Buffer AVL/ carrier RNA proportionally.*

2. Add 140µl of plasma to the bufferAVL /Carrier RNA in the micro centrifuge tube. Mix by pulse vortexing for 15sec. (*To insure efficient lyses, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogenous solution. Frozen samples that have only been thawed once can also be used).*

3. Incubate at room temperature (15-25c) for 10 min.(Viral particle lysis is complete after lysis for 10 min at room temperature. *Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and the Rnases are inactivated in buffer AVL).*

4. Briefly centrifuge the 1.5ml micro centrifuge tube to remove droplets from the inside of the lid.

5. Add 560µl of ethanol (96-100%) to the sample, and mix by pulse vortexing for 15sec.After mixing, briefly centrifuge the 1.5ml micro centrifuge tube to remove drops from the inside of the lid.(*Only ethanol should be used since other alcohols may result in reduced RNA yield and purity .if the sample volume is greater than 140µl, increase the amount of ethanol proportionally. In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogenous solution*.)

6. Carefully apply 630µl of the solution from step5 of the QIAamp spin column (in a 2ml collection tube0 without wetting the Rim. Close the cap, and centrifuge at 6000x g (8000rpm) for 1 min. Place the QIAamp spin column into a clean 2ml collection tube and discard the tube containing the filtrate.*(Close each spin column in order to avoid*

   cross contamination during electrophoresis. Centrifugation is performed at 6000-x g in order to limit micro centrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA.If the solution has not yet been completely passed through the membrane, centrifuge again at a full speed until all of the solution has passed through.)

7. Carefully open the QIAamp spin column, and repeat the step 6.

8. Carefully open the QIAamp spin column, and add 500µl of Buffer AW1.Close cap and centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp spin column in a clean 2ml collection tube and discard the tube containing the filtrate.

9. Carefully open the QIAamp spin column, and add 500µl of Buffer AW2. close the cap and centrifuge at full speed (20,000 xg; 14,000prm) for 3 min.Continue directly with the step 10, or to eliminate any chance of possible Buffer AW2 carryover, perform step 9a,and then continue with step 10**.(***Residual Buffer AW2 in the elute may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, and contacting the QIAamp spin column. Removing the QIAamp spin column and the collection tube from the rotor may cause flow-through to come into contact with the QIAamp spin column. In these cases, the optional step 9a should be performed.)*

   9a. Place the QIAAmp spin column in a 2ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. Place the QIAamp spin column in a clean 1.5ml micro centrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and 60µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000rpm) for 1 min.(*A single elution with 60µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp spin column. Performing a double elution using 2 x 40µl of Buff rave will increase yield by upon 10%. Elution with volumes of less than 30µl will lead to reduced yield and will not increase the final concentration of RNA in the elute.)*

Viral RNA is stable for up to 1 year when stored at –20º c or -70ºc.

4.2 Reverse Transcription & Amplification

Reverse Transcription (RNA – cDNA)

5µL of extracted RNA is added to 15µL of RT mix in a 0.2ml PCR tube. This is for one sample.

- Reagent control: For a set of samples there should be one reagent control as a part of QC. 5µL of sterile molecular grade water is added to 15 ul of RT mix

- Master Mix Preparation (MMX): RT Mix
  The RT mix should consist of the following reagents with specified concentrations.
  1X Qiagen PCR buffer, 3mM Mgcl2, 500µM of each dNTP (MBI), 250ng random hexamers (promega), 10U RNAsin (Ambion) and 150 U superscriptII (MBI Fermentas).
  Refer Appendix I for RT MIX preparation

- RT Program: [25^{0}C 10 min], [42^{0}C 1 h], [70^{0}C 15 min], [4^{0}C hold]

Amplification – PCR

Amplification of two regions of the *pol* gene, protease (PI)& the Reverse transcriptase (RT) regions are amplified using gene specific primers.

- Reagent control: For each reaction there should be a reagent control.Add 3µL of Sterile molecular grade water to 27µL of each RT & PI MMX.

- Amplification of Protease & RT region
  3µL of c DNA is added to 27µL of master mix containing PR & RT specific primers, per sample.

- Master Mix preparation:

  The master mix contains 1x qiagen PCR buffer, 1.5mM MgCl2 (do not add this as it is already present in the buffer), 200µMof each dNTP, 0.4µM of each sense) & anti sense primers specific for each regions, and 2.5 units of DNA polymerase.

NOTE : Refer Appendix II for MMX preparation for PI & RT reactions

- Thermocycler Program for First Round PI & *RT*

PCR:	Primers:	Program:	
1st round for PR	PT-1/PT-2	95^{0}C 1min	[95^{0}C 15s/65^{0}C 30s/72^{0}C 45s] x 35
1st round for RT	R-1/R-6	95^{0}C 1min	[95^{0}C 15s/60^{0}C 30s/72^{0}C 1min] x 35

Detection

- Analyze PCR products on 1% agarose gel (***preparation given in section 4.5***)
- If band of 400 bp is visualized, pack the product ***(refer section 5)*** & send it for purification & sequencing.
- If no band is visualized go for nested PCR of the gene.

NESTED PCR: Add 1µL of first round PCR product to 49µL of master mix**.** The reaction volume here is 50*µl*

.

Note: **Refer Appendix III for MMX preparation for PI & RT Nested PCR reactions.**

Thermocycler Program

PCR:	Primers:	Program:	
2nd round for PR	PT-2/PT-3	95^0C 1min	[95^0C 15s/55^0C 30s/72^0C 45s] x 25
2nd round for RT	R-3/R-6	95^0C 1min	[95^0C 15s/55^0C 30s/72^0C 1min] x 25

Detection: Presence of Amplicon is checked on an agarose gel .If visible, send the product for purification & sequencing. If not, repeat the test.

ELECTROPHORESIS.

The PCR products are analyzed on 1% agarose gel to check if the specific product is visible.

Buffer preparation

Available Stock – 50 x TAE (Tris acetic acid EDTA)
Working Stock: 1X

Mix 20ml of 50X buffer & make up to 1000ml with sterile deionised water. Mix well.
Reconstituted buffer is stable for 2 weeks at room temperature.

Preparation of 1% agarose gel;

a) Operation of electronic balance**:**

- Switch on the mains & the electronic balance.
- Wait till it comes to the weighing mode. (0.00g)
- Place a piece of butter paper, close the glass door & Press the T button (TARE button)
- Wait till it comes to 0.00g
- Weigh 0.3 grams of agarose by opening the side door.
- Remove the contents. Wipe the surface with tissue.
- Press TARE, wait till it shows zero.
- Switch off.
- Sign in the logbook placed beside the balance.

b) 1% Agarose gel Gel preparation

- Add 30ml of 1X TAE buffer into a clean 250ml conical flask.
- Add the weighed agarose to the flask
- Heat the mixture in microwave (Area 1) for 30 sec

c) Microwave operation:

- Switch on the instrument, takeout the beaker with water placed inside the microwave
- Place the flask, close the door, & press the " 30 " sec button
- On completion a beep sound is heard, Remove the hot flask carefully.
- Keep the door open for few minutes to cool.
- Replace the beaker with water inside the microwave & close the door.
- Switch off.
- Add 2.7 µL of ETBr (Ethidium Bromide, 10g/ml) to the molten agarose & mix.
- Allow to cool to 50 – 60 ºC.

Electrophoresis

a) Arranging the unit.

- Wash the electrophoresis tank,
- Place the gel tray in the middle of the tank,
- Slide the gel casting gates into the slots at the opposite ends of the gel tray.
- Place the combs into the appropriate slots of the base so that the sample wells are near the cathode (black). ***DNA samples will migrate towards the anode (red) during electrophoresis as they are negatively charged.***
- Poor the molten agarose (cooled to 50- 60ºC.) between the gates.
  (Remove any bubbles formed during pouring with a pipette tip)

- Allow 20 – 40 min for the gel to solidify at room temperature.(22- 25 ºC.)
- Carefully remove the comb from the solidified gel. Remove the gel casting gates.
- Submerge the gel beneath 2 to 6 mm of 1X TAE buffer.
- Replace the combs & gel casting plates into their respective positions after wiping.

b) Sample Loading & Electrophoresis.

After the agarose gel has solidified. Sample loading & electrophoresis can begin.

- Take a piece of paraffin .Mix 1µL of 6X loading dye with 5µL of sample (DNA).
- Load the samples into the wells using standard pipettes.
- Close the lid, & attach the red & the black cords respectively (from the unit to the power pack).
- Set the temperature in the power pack to 60- 70ºC for 30 min. Start the power back.

UV Tans illuminator:

Once the electrophoresis is over, remove the cords, open the lid, & place the Gel on a plastic wrap on the UV Tran illuminator for nucleic acid visualization & analysis. DNA - ETBr complexes may be illuminated with UV light of 254, 302 or 366nm.

GEL DOCUMENTATION SYSTEM

Document the gel on the gel documentation system, present in Area 3 (serology section).

- Switch on the **Universal Hood System**

- Turn on the universal Hood main switch (on the rear left side of the cabinet).
- Select the **acquisition mode** from the file menu.
- Either open the front door or open the drawer of the hood.
- Position the gel and close the door.
- Press the **Epi-Illumination Key** to turn on the epiwhite light.
- Adjust the lens aperture, zoom, and focus while looking at the computer Screen.
- Open the door and reposition the gel if necessary.
- Press the **UV Trans Key** to turn on the UV light.
- Adjust the iris, focus & zoom till you get the picture you want.
- Save the picture
- Press, "annotate" to label the gel picture. Save & print
- Take the Gel out and wipe the surface of the acrylic sheet with dry tissue paper. The surface of the Trans illuminator must be wiped with spirit and wipe it dry.
- Close the Hood door & switch off the instrument.
- Documentation: Testing Personnel must enter his/her signature with date in the Gel Doc Log Book.

Gel Doc system - Trouble Shooting

Problem	Possible cause	Solution
Image is not visible on the monitor	Aperture is closed	Open the aperture
-do-	Incorrect monitor settings.	Refer the computer manual
-do-	Wrong cable connections.	See appendix A or B for correct cabling.
Image is not bright enough.	Wrong aperture setting.	Open the aperture.
Impossible to image whole sample area.	Lens is Zoomed - in too close.	Zoom out the lens.
Print out does not look the same as the image on the monitor.	Monitor settings are wrong.	-do-
Print out area is not the same as the image on the monitor.	Improper dipswitch selection on the printer.	The dipswitch must be 1 and 8up,and rest down.
Light leakage into the darkroom.	The rear hole on the MZL not closed with the screw.	Close the hole with the black plastic screw.

5. PACKING OF SAMPLE TO BE SHIPPED

5A) *Materials required*:

- Autoclaved 0.5ml eppendoff tubes
- Para film
- Labels

- Cellophane tape
- Small box for packing

5B) *primer preparation:*

- Required concentration; 5picomoles/µL
- Volume /sample: 10 µL
- Sequencing primers: **PT3** for protease & **R3** & **R7** for Reverse Transcriptase

Prepare primers at the specified concentrations & aliquot them in o.5 ml labelled eppendoff tubes. Close the tube & Wrap the lid with paraflim. Apply a piece of cellophane tape on the label to keep it in place.

5C. *Sample packing*:

Aliquot 20µL of each sample in a sterile autoclaved 0.5 ml labeled eppendorf tubes. Close the tube& wrap the lid with Para film. Apply a piece of cellophane tape on the label to keep it in place.

Pack all the primers & sample in the appropriate box.

5D. *Package list:*

Type the list of samples Ids to be purified & sequenced. Mention the names of appropriate primers to be used for PI & RT sequencing. Place the list in the box & ship the box to the following address:

Mr.TILAK WAG RAY,
ALCHEMY DIAGNOSTICS Pvt.Ltd.
Regd .off: 3-4-802/G-5, Mahathi Apartments,
Barkathpura, Hyderabad- 500027,
A.P, India.
Tel.No 91 40 27567030, 55755844
Mobile: 33768139.

5E) Document for Shipping:

Get a document certifying the samples to be shipped As non infectious 7 get it signed by the Lab Manager. This has to be handed over to the courier service along with the sample pack. Mention the no of vials & volume being shipped in the letter.

Note: Refer Appendix IV for a sample of the letter.

QUALITY CONTROL

One of the important concerns in molecular work is amplicon contamination, which affects quality of the sequences amplified & lead to false positive results. The following procedures have to be followed strictly for better quality & correct results.

6a) Dedicated use of Equipments:

- Separate coolers are to be used during reagent preparation & sample loading. The coolers used for **reagent preparation** are placed inside the **-20ºC** freezer & the cooler for **sample loading** is placed in **fridge "3"** freezer compartment, at the entrance.
- Pipettes, minifuge & vortexes are to be dedicated to each Area. Don't interchange.
- Don't prepare reagents outside the reagent preparation hood. Wipe the Hood with spirit after each use & document in the log placed above the hood.
- Store the amplicons only in the **fridge "3"** at the entrance.
- Don't exchange coats or consumables between different areas.
- Always move in an unidirectional way, from reagent preparation, sample loading, amplification & detection .

6b) .Use Gloves:

- Don't open the - 20ºCFreezer with out gloves to maintain a **RNAse free Zone**
- Do not touch the reagents or freezer with the gloves used in the detection area to prevent reagent contamination with amplicons.

6c). Reagent Control:

- Always include a reagent control, containing the MMX & water to check for any reagent contamination during each reaction.

6d) .Swipe Test:

Swipe test is done monthly to check for environmental contamination. Refer swipe test SOP for the same.

6e) Use of DNA ZAP Solution:

DNA ZAP inactivates any amplicons on the work surface or instrument surface.

- Spray solution 1 & solution 2 simultaneously, wait for 3 minutes.
- Wipe dry with tissue.
- Rinse with Distilled water.

DNA Zap can be used in case of Amplicon spill.

APPENDIX I

Preparation of Master Mix for the conversion of RNA to CDNA

CONTENTS REQUIRED	1x	2x	4x	6x	8x	10x
QIA buffer	2µl	4µl	8µl	12µl	16µl	20µl
DNTPS	1µl	2µl	4µl	6µl	8µl	10µl
RNASIN	1µl	2µl	4µl	6µl	8µl	10µl
Random Primers	0.5µl	1µl	2µl	3µl	4µl	5µl
RH minus	0.75µl	1.5µl	3µl	4.5µl	6.0µl	7.5µl
$Mgcl_2$	1.2µl	2.4µl	4.4µl	7.2µl	9.6µl	12µl
Total Amount of Master Mix	-	10.5µl	25.8µl	38.7µl	51.6µl	64.5µl
Total Amount of Water	-	15x2=30µl 30-10.5=**19.5µl**	15x2=60µl 60-25.8=**34.2µl**	15x6=90µl 60-38.7=**51.3µl**	15x8=120µl 120-51.6=**68.4µl**	15x10=150 150-64.5=**85.5µl**

Appendix II
Master Mix Preparation for the amplification of Protease Region
Total Reaction Volume 30µl

CONTENTS REQUIRED	**1x**	**2x**	**4x**	**6x**	**8x**	**10x**
Buffer	3µl	6µl	12µl	18µl	24µl	30µl
DNTPS	0.6µl	1.2µl	2.4µl	3.6µl	4.8µl	6µl
Primer 1 (PT1)	0.24µl	0.48µl	0.96µl	1.44µl	1.92µl	2.4µl
Primer 2 (PT2)	0.24µl	0.48µl	0.96µl	1.44µl	1.92µl	2.4µl
Biotool DNA Polymerase	0.15µl	0.30µl	0.6µl	0.9µl	1.2µl	1.5µl
Total Amount of Master mix	-	8.46µl	16.92µl	25.38µl	33.84µl	42.30µl
Total Amount of Water	-	27x2=54µl 54-8.43=**45.54 µl**	27x4=108µl 108-16.92=**91.08 µl**	27x6=162µl 162-25.38=**136.62µl**	27x8=216µl 216-33.84=**182.16µl**	27x10=270µl 270-42.30µl=**227.7µl**

Master Mix Preparation for the amplification of RT Region
Total Reaction Volume 30µl

APPENDIX III

Master mix Preparation for nested PCR of protease gene
Total reaction volume – 50µl

CONTENTS REQUIRED	1x	2x	4x	6x	8x	10x
Buffer	5µl	10µl	20µl	30µl	40µl	50µl
DNTPS	1µl	2µl	4µl	6µl	8µl	1µl 0
Primer 1 (PT2)	0.4µl	0.8µl	1.6µl	2.4µl	3.2µl	4µl
Primer 2 (PT3)	0.4µl	0.8µl	1.6µl	2.4µl	3.2µl	4µl
DNA Polymerase	0.25µl	0.5µl	1µl	1.5µl	2µl	2.5µl
Total Amount of Master mix	-	14.1µl	28.2µl	42.3µl	56.4µl	70.5µl
Total Amount of Water	-	49x2=98µl 98-14.1=**83.9µl**	49x4=196µl 196-28.2=**167.8µl**	49x6=294µl 294-42.3=**251.7µl**	49x8=392µl 392-56.4=**335.6µl**	49x10=490µl 490-70.5=**419.5µl**

Master mix preparation for nested PCR of RT gene

Total Reaction Volume - 50µl

CONTENTS REQUIRED	**1x**	**2x**	**4x**	**6x**	**8x**	**10x**
Buffer	5µl	10µl	20µl	30µl	40µl	50µl
DNTPS	1µl	2µl	4µl	6µl	8µl	1µl 0
Primer 1 (R3)	0.4µl	0.8µl	1.6µl	2.4µl	3.2µl	4µl
Primer 2 (R6)	0.4µl	0.8µl	1.6µl	2.4µl	3.2µl	4µl
DNA Polymerase	0.25 µl	0.5µl	1µl	1.5µl	2µl	2.5µl
Total Amount of Master mix	-	14.1µl	28.2µl	42.3µl	56.4µl	70.5µl
Total Amount of Water	-	49x2=98µl 98-14.1=**83.9µl**	49x4=196µl 196-28.2=**167.8µl**	49x6=294µl 294-42.3=**251.7µl**	49x8=392µl 392-56.4=**335.6µl**	49x10=490µl 490-70.5=**419.5µl**

HPTN
OPERATING POLICY

OP No.: HPTN016-01
Title: QA and Verification of HIV Test Results
Effective Date: 01 Aug 2006

APPROVAL

Chairperson, HPTN Prevention Leadership Group Date

PURPOSE

As a site specific quality assurance measure to verify the HIV infection status of clinical trial participants, the HPTN Central Laboratory (CL) will perform the relevant protocol related testing at the end of enrollment. Specimens from seroconverters and equal number of HIV negative participants will also be tested to verify site results. This testing will be done to verify local laboratory test results and in special circumstances, samples tested at a non HPTN centralized location (local commercial laboratory). The HPTN CL will use the same test method as used for the original test. Discrepancies may be resolved using test methods with different sensitivities.

SCOPE

1. Baseline plasma/serum samples from 50 or ten percent (whichever is greater) of randomly selected enrolled adult subjects at each site will be retested for HIV antibody by the HPTN CL using FDA licensed tests. Samples from all subjects will be retested if there are less than 50 total trial subjects. In the event of a false positive or false negative result that changes the infection status of the subject, an additional 100 or 20% of samples (whichever is greater) from enrolled subjects will be retested.

2. Baseline and seroconversion plasma/serum samples from all seroconverting adult subjects and an equal number of randomly selected samples from uninfected subjects matched by follow-up visit will be retested by the HPTN CL using FDA licensed tests (i.e., HIV antibody, HIV DNA PCR or HIV RNA if necessary). If not otherwise specified in the protocol, specimens will be retested at the end of the study. In the event of an *unexpected result, i.e. positive baseline sample or negative end point sample in a seroconverter, retesting of additional aliquots or time points may be performed* **as determined by the HPTN CL.**

3. Plasma/serum samples from all HIV infected infants in perinatal trials and an equal number of randomly selected uninfected infants will be retested by the HPTN CL using FDA licensed tests (i.e., HIV antibody, Roche Amplicor 1.5v HIV RNA) or the Roche Amplicor HIV DNA PCR assay).

RESPONSIBILITIES

The SDMC is responsible for:

- Generating PTIDs for retesting
- Providing retest PTIDs to the sites
- Providing PTIDs and HIV test results from participant CRFs to the HPTN CL

The HPTN CL is responsible for:

- Working with sites to ship samples to CL for retesting
- Conducting the retesting
- Providing the SDMC with all discrepant results resulting from the retesting

PROCEDURES

1. Retest PTID generation and distribution
  1.1. The SDMC provides the HPTN CL with regular updates on study enrollment status and seroconverters.
  1.2. The SDMC notifies the HPTN CL when retesting is due for a protocol.
  1.3. The SDMC generates a retest list containing PTIDs and associated specimen collection dates for retesting following the guidelines specified under "SCOPE" and sends the list to the HPTN CL and to the site(s) along with instructions to pull and ship specimens to the CL.

2. Retesting
  2.1. Site(s) pull and ship specimens to the HPTN CL using the PTIDS and collection dates.
  2.2. The HPTN CL conducts the retesting and informs the SDMC when retesting has been completed.
  2.3. The SDMC provides a retest list to the HPTN CL containing retest PTIDS, collection dates, and the HIV test results performed at the site(s) local laboratories and documented by the site(s) on study CRFs.
  2.4. The HPTN CL matches the HIV retest results to the site(s) local laboratory results and identifies any discrepancies. The HPTN CL and SDMC will follow up on discrepancies as appropriate.
  2.5. Following completion of study retesting, the HPTN CL sends a report to the SDMC that contains:
    2.5.1. PTIDs with discrepant results, associated visit codes, and collection dates
    2.5.2. PTIDs with results that were unavailable for retesting with associated visit codes and collections dates

3. Data Management
  3.1. The SDMC files the discrepant result report and;
  3.2. Incorporates and documents the retest result.

DEFINITIONS
Definitions of terms referenced within the context of this document can be accessed in the Glossary section of the HPTN Manual of Operations (MOP).

APPENDICES
Not applicable

REFERENCES
Not applicable

HPTN
OPERATING POLICY

OP No.: HPTN019
Title: HIV Endpoint Determination
Effective Date: 01 MAR 2006

APPROVAL

Chairperson, HPTN Prevention Leadership Group Date

PURPOSE
To define considerations and procedures associated with confirmation of HIV endpoints in HPTN studies.

SCOPE
This procedure applies to all HPTN organizations, investigators, and staff members involved in conducting HPTN studies with HIV endpoints.

RESPONSIBILITIES

1. HPTN Protocol Teams are responsible for specifying HIV testing algorithms in HPTN study protocols that are scientifically appropriate for the study population and study objectives.
2. HPTN Investigators of Record (IoRs) are responsible for ensuring that protocol-specified HIV testing algorithms are followed when conducting HPTN studies.
3. HPTN Central Laboratory (CL) is responsible for performing quality assurance and confirmatory HIV testing for HPTN studies as specified in HPTN study protocols.
4. HPTN Endpoint Adjudication Committee is responsible for providing guidance to HPTN Protocol Teams with regard to determination of HIV endpoints. Decisions of the committee are considered final for purposes of primary analyses of HIV endpoints.
5. HPTN Statistical and Data Management Center (SDMC) is responsible for providing data reports to the HPTN Endpoint Adjudication Committee; convening committee meetings; documenting committee meetings and decisions; and incorporating committee decisions into HPTN study databases.

PROCEDURES

1. As part of the protocol development process, HPTN Protocol Teams will specify HIV testing algorithms in study protocols that are scientifically appropriate for the study population and study objectives. HPTN IoRs will make every effort to ensure that protocol-specified HIV testing algorithms are followed throughout the period of study implementation. The HPTN CL will perform quality assurance and confirmatory HIV testing as specified in study protocols.

2. The HPTN Endpoint Adjudication Committee will be composed of the Principal Investigator of the HPTN CL and four additional members with experience and expertise in HIV testing. A subset of at least three committee members will be assigned to perform committee functions for each HPTN study. For each study, the assigned committee members will have no scientific affiliation with the study (e.g., Protocol Team members may not serve as committee members). The Principal Investigator of the HPTN CL will chair each study-specific committee unless he/she has a scientific affiliation with the study. Protocol Team members including DAIDS PSB representatives and study operations staff from the HPTN CORE, SDMC, and CL may take part in committee meetings as non-voting discussants or observers.
3. It is not necessary or expected that the Endpoint Adjudication Committee will review all HIV endpoints for all HPTN studies with HIV endpoints. Rather, the Endpoint Adjudication Committee will provide guidance to Protocol Teams in the event that one or more study participants' final HIV status is not unequivocal or the time point at which one or more participants became infected is not clear. For example, it is expected that issues or questions related to HIV endpoint determination will arise if:
   3.1. a protocol-specified HIV testing algorithm is not followed
   3.2. HPTN CL test results do not confirm Local Laboratory test results
   3.3. indeterminate test results persist at study exit
   3.4. an unusual pattern of test results is observed

   HPTN Protocol Teams will refer all issues and questions related to HIV endpoint determination to the Endpoint Adjudication Committee. The HPTN SDMC Statistician for each study (or designee) will provide data reports to the Endpoint Adjudication Committee as needed to support review and decision-making by the committee. For blinded studies, data provided to the committee will not include participants' treatment assignments.

4. For each study, the Endpoint Adjudication Committee will develop written "terms of reference" to guide their review and decision-making. The terms of reference will specify, for example, considerations related to deviations from protocol-specified testing algorithms and discordance between CL and Local Laboratory test results. The terms of reference also will specify the membership of the committee for the study, procedures for communication with the Protocol Team, and the format and frequency of Endpoint Adjudication Committee meetings. Terms of reference must be finalized for each study prior to undertaking any data reviews and decision-making for that study.
5. Designated staff from the HPTN SDMC will provide administrative support to the Endpoint Adjudication Committee. These staff will arrange and convene committee meetings and document committee meetings and decisions.
6. Decision-making by the Endpoint Adjudication Committee will be considered final.
7. HPTN SDMC Statisticians will incorporate committee decisions into HPTN study databases for purposes of HIV endpoint analyses.

DEFINITIONS

None

Section 15: Peripheral Blood Mononuclear Cells

1. Overview: Peripheral blood mononuclear cells (PBMC) are collected in certain studies for HIV cultures, obtaining viral isolates as well as storage for future experiments to test cellular immune responses against HIV, identify cytokine or chemokine secretion patterns, and isolate and characterize viruses. These assays require PBMCs that have been isolated and cryopreserved under strictly defined conditions to ensure optimal recovery, viability, and functionality. There are numerous methods for PBMC isolation and cryopreservation.

   This section contains two example SOP's for PBMC; there are many different procedures for PBMC isolation. It is essential to review these SOP's carefully and discuss the requirements for your site with the HPTN and/or MTN Central Laboratories to ensure that the procedure is correct for your needs.

   Training can be found at: http://www.ccghe.org/pbmc/player.html

2. Specimen: EDTA, heparin, or ACD anti-coagulated whole blood can be used. Processing should occur within 30 hours of collection of sample, typically within 2 – 4 hours of collection. In most adult populations, the expected yield of PBMC is approximately $1.5x10^6$ cells/mL with a range of $0.8 – 3.0x10^6$ cells/mL after gradient purification. Each protocol will define the viable cell number requirements.

3. Internal Quality Control: Cell yields determined after cell isolation and after freezing for recovery are used for quality control. Acceptable limits and systems for monitoring yields must be established.

4. External Quality Control: At the present time, there is no international DAIDS approved proficiency program. The IQA is responsible for the domestic quality assurance for certain domestic trials while the VTN has quality assurance monitors in place for their trials.

5. Calibration: Appropriate maintenance procedures for laboratory equipment such as pipettes and centrifuges are needed.

6. Specimen analysis: These PBMC procedures are for the isolation and storage of cells. Other techniques such as ELISpot are required for the analysis of the cells.

7. Microscopy: Hemacytometers using light field microscopy are used for manual counting of cells and for the determination of cell viability.

8. Troubleshooting Specimens: Samples must be checked for clots before use-do not use clotted samples.

9. Maintenance: appropriate maintenance procedures for laboratory equipment such as timers, pipettes and bio safety airflow hoods are needed.

SOP for PBMC Isolation and Cryopreservation

Cross-Network version 1: 04 November 2005

Owner(s), Name and Title	

Approved By	Name, Title	Signature	Date

Revision History	Version # [0.0]	Effective Date [ddmmmyy]	Description (notes)

Distributed Copies to	Name (or location)	# of copies	Name (or location)	# of copies

SOP Document Annual Review	Name, Title	Signature	Date

Section 15: PBMC Testing

Purpose

This Standard Operating Procedure (**SOP**) describes a procedure for the isolation and cryopreservation of Peripheral Blood Mononuclear Cells (**PBMC**) from whole blood.

Scope

This procedure is to be utilized for processing blood samples for the isolation, cryopreservation, and storage of PBMC samples obtained for DAIDS network protocols.

Background

Freshly collected or cryopreserved PBMC are used for evaluation of vaccine or antiretroviral therapy-induced cellular immune responses, HIV-associated changes in immune response, and recovery of replication competent virus. Validation studies conducted across networks indicated that blood must be processed and frozen within 8 hours (or as defined in a specific protocol) from the time of blood draw to maintain maximum function of the cells in immune-monitoring assays. These assays require PBMC that have been isolated and cryopreserved under strictly defined conditions that ensure optimal recovery, viability, and functionality.

Authority and Responsibility

1 The Associate Director (or his/her designee) of Laboratory Operations, the Laboratory QA Program Manager and the International Laboratory Program Manager have the authority to establish this procedure.
2 The central Laboratory Program and the local site Quality Assurance Units are responsible for the control of SOP documentation.
3 The Principal Investigator/Laboratory Manager is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.
4 All technicians working on DAIDS network studies are responsible for reading and understanding this SOP prior to performing the procedures described.

Specimen

1. Patient Preparation

None

2. Specimen Type

Sodium heparin-, EDTA- or ACD-anti-coagulated whole blood (as specified by protocol).

3. Optimum/Minimum Specimen Volume

Required blood volume determined by protocol

4. Handling Conditions

4.1 Fresh, anti-coagulated whole blood specimens should be stored at room temperature (1530°C) from the time of collection until delivery to the laboratory/processing unit.

4.2 Do not refrigerate or freeze whole blood.

4.3 If specimens are received at temperatures below 15°C, allow whole blood to come to room temperature (15-30°C) before processing.

4.4 The use of a processing worksheet is strongly recommended to track the timing of processing and document problems that arise during processing. Examples of PBMC processing worksheets may be viewed in Appendix I. Record the time that processing began on the PBMC Processing Worksheet or other data collection record (e.g. LDMS).

4.5 If some of the specimen blood tubes contain small clots, try to remove the clots prior to processing. Record the total number of tubes that contained small clots in the comments section on the PBMC Processing Worksheet (refer to the QC section for additional information on the worksheet.).

5. Unacceptable Specimens

5.1 Unlabeled or mislabeled specimens will be rejected.

5.2 Grossly hemolyzed (4+) specimens should not be used for PBMC.

5.3 If a specimen tube is grossly clotted (see glossary), remove that tube from the specimen batch and process the remaining tubes.

5.4 Record the total number of tubes that were discarded due to gross clots or gross hemolysis in the comments section on the PBMC Processing Worksheet.

5.5 Document all unexpected specimen conditions on the PBMC Processing Worksheet (or whatever method of tracking is defined by the protocol/network) and enter the information into a laboratory data management system, such as the Laboratory Data Management System (LDMS).

Equipment

Recommended vendors and equipment are listed. Unless otherwise specified, equipment of equal

or better quality than those recommended can be used.

1. Preparation & Processing

1.1 Biological laminar flow safety cabinet, as set up by laboratory (P2, P2.5 or P3)
1.2 Centrifuge, low-speed (capable of 300 to 1000 x g), 18-22 °C refrigerated preferred, ambient acceptable
1.3 Micropipettes, range 20, 200, 1000µL
1.4 Pipette-Aid (cordless preferred) for disposable, serological pipets
1.5 2-8°C refrigerator
1.6 –20°C freezer (for FBS storage)
1.7 –70/-80°C freezer (for short term PBMC storage)
1.8 37-56°C water bath
1.9 Bleach bucket

2. Liquid Nitrogen Storage Equipment (use dependent on network storage/shipping instructions)

2.1 Liquid nitrogen storage tank (≤ –140°C)
2.2 Liquid nitrogen dry shipper

3. Cell Counting (select one of following options)

3.1 Automated cell counter (Beckman-Coulter, Abbott CelDyn, Sysmex, Guava or equivalent)
3.2 Manual cell counting chamber (Neubauer hemacytometer) with Light-field microscope

4. Cryopreservation (use one of following options)

4.1 StrataCooler must be at 2-8°C before starting the cool down of the cryovials. Do not place cryovials in containers cooled to temperatures below 2°C.
4.2 Mr. Frosty, 1 °C cryo-freezing container (Nalgene). Mr. Frosty must be room temperature before use. *Note:* Mr. Frosty requires an explosion proof freezer for use. Alcohol should be changed after every fifth freeze/thaw cycle – a log should be used to track freeze thaw/cycles and reagent changes.
4.3 Cryomed Freezing Chamber (Gordinier)

Disposables

Recommended vendors and brands are listed. Unless otherwise specified, products of equal or better quality than those recommended can be used.

1. Plastics

1.1 Serological pipets, disposable, 5, 10, 25 & 50mL, sterile, individually wrapped
1.2 Sterile precision pipet tips, 20, 200, 1000 µL
1.3 15 and 50mL disposable centrifuge tubes, sterile, conical bottom, graduated, polypropylene

1.4 Sterile bottles/flasks, disposable, 45mm neck, Polystyrene, 250mL

1.5 Cryogenic vials (**cryovials**), 1.8-2mL, externally threaded screw cap with o-ring, sterile, Polypropylene only, self-standing, graduated, leak-proof, formulated for vapor-phase liquid nitrogen preservation (-190 °), 12.5x49mm (Nalgene Labware Brand Products, catalog # 50000020; Wheaton, catalog #985734; Fisher Scientific, catalog #05-669-57; Corning, catalog #430659 (12.7x49mm), Sarstedt, #72.694.006)

Note: Not all cryovial brands are suitable for long-term storage in liquid nitrogen. Check with your network prior to substitution of this item. For Merck protocols use Sarstedt cryovials only.

1.6 Optional: 5mL sterile, individually wrapped plastic transfer pipettes

2. Markers

Markers for writing on processing tubes and vials should have a fine point, and contain fast drying, indelible ink. (Example: Fisher Scientific, fine line, felt tip making pen, order #S32179).

3. Labels

Cryogenic labels suitable for -70 °C and LN2 temperatures meeting LIMS and network specifications. Examples: Cryo-Tags and Cryo-Babies from Diversified Biotech. Brady B461, B490.

Personal Protective Equipment

Personal protective equipment suitable for use with Blood Borne Pathogens is required. Follow your laboratory guidelines and practices for the handling of blood products.

1 **Laboratory coat**
2 **Eye protection**
3 **Latex or nitrile Gloves, non-powdered is preferred, powdered is acceptable**

Reagents

Recommended vendors are listed. Unless otherwise specified, reagents of equal or better quality than those recommended can be used.

1. Blood Separation Reagents

Wash Reagents

1.1 Hanks' Balanced Salt Solution (**HBSS**) without calcium or magnesium, ready-to-use (Gibco/BRL Life Technologies, Grand Island, NY, USA, Tel. 1-800-828-6686)

1.2 Alternative: 1X Phosphate buffered saline (PBS) without calcium or magnesium. Note: Store opened bottles at room temperature (15-30°C) until used or until

manufacturers expiration date. Discard if visible signs of contamination, such as cloudy appearance, develop.

1.3 Merck only: HBSS with 5% FBS, expiration 1 month, store at 2-8 ^{o}C

Cell Separation Tube with Frit Barrier (CSTFB)

1.4 Accuspin System-Hypaque-1077 cell separation tubes with frit (barrier), ready-to-use, 50mL tubes with 15mL Histopaque (Sigma)

Storage conditions:

- Store in the refrigerator (2-8 ^{o}C)
- Protect from light
- Check the manufacture's expiration date.
- Tubes shipped at ambient temperature are stable for up to 3 weeks. Refrigerate upon arrival.
- A cloudy appearance indicates deterioration of the product.
- Allow the Accuspin tubes to come to room temperature (15-30°C) prior to use.

1.5 Alternatives to pre-filled Accuspin System:

Dry Accuspin separation tubes

Dry Leucosep separation tubes

Lymphoprep (prefilled) separation tubes, Greiner Bio-one (Axis-Shield, Norway)

Dry Accuspin separation tubes plus gradient media*

Density Gradient Media for Cell Separation Tubes

Sigma Histopaque 1077

Amersham Biosciences Ficoll-hypaque

Axis-Shield Lymphoprep media (Greiner Bio-one)

Lysing Reagents (Merck Only)

1.6 ACK lysing Buffer, formula #79-0422DG, Gibco BRL. Store ambient (15-30C).

Note: No substitution, use Merck specific formula only.

2. Freezing Reagents

2.1 Fetal Bovine Serum (**FBS**), heat inactivated preferred (Gemini Bioproducts, or as specified by
your network).
Check with your network prior to vendor substitution. Not all brands of FBS are equivalent.
Issues regarding quality control, toxicity, and background must be addressed before changing
vendors.

Obtain a certificate of analysis from the vendor for your quality control records.

Note: A copy of the FBS certificate of analysis may be required to export (or import) PBMC
aliquots between countries. Some country restrictions apply depending on the source of origin.
FBS stored frozen (≤ -20°C) is good until the manufacturer's expiration date.
Thawed and stored at 2-8°C, stability is one month.

2.2 Dimethylsulfoxide (**DMSO**) (Sigma Chemical Co., catalog #D5879 or D2650)
Store unopened bottles at room temperature. Unopened bottles are stable for an indefinite

period of time.
After opening, undiluted DMSO is stable at room temperature (15-30°C) when protected from
moisture, for 2 years.

Use aseptic technique when removing DMSO from the bottle to avoid possible contamination.
Discard open bottle if visible signs of contamination are noted.

3. Disinfectant

3.1 70% Ethanol Disinfectant, spray bottle

3.2 10% Bleach, bucket and spray bottle

3.3 Other disinfectant as specified by your laboratory policy

Reagent Preparation

1. Heat Inactivated FBS (HI-FBS)

Heat inactivated FBS can be ordered from the manufacturer. If you receive heat inactivated FBS, follow these instructions for thawing, aliquoting and use of FBS.

1.1 Remove FBS from the -20°C freezer.

1.2 Thaw overnight in the refrigerator (2°-8°C), preferred, or for several hours at room temperature. Do not allow FBS to sit at room temperature any longer than necessary to complete the thawing process.

1.3 Gently swirl two or three times as the FBS thaws.

1.4 Once thawed, mix the FBS gently but thoroughly and using aseptic technique, aliquot into sterile, labeled 50mL conical tubes.

Note: Labels should identify these tubes as " HI- FBS" and include the lot number, the aliquot date, the expiration date, and your initials.

1.5 Refrigerate (2°-8°C) the number of aliquot tubes you need for your monthly expected workload. Mix well before use. Refrigerated FBS is stable for one month.

Note: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

1.6 The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacturer's expiration date.

1.7 To use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well before use.

2. FBS Heat Inactivation Preparation

If FBS has is not heat inactivated by the manufacturer, heat inactivate using the instructions below.

2.1 Remove FBS from the -20°C freezer.

2.2 Thaw overnight in the refrigerator (2°-8°C), preferred, or for several hours at room temperature. Do not allow FBS to sit at room temperature any longer than necessary to complete the thawing process.

2.3 Gently swirl two or three times as the FBS thaws.

2.4 Place FBS in a 56°C (55– 57°C) water bath. Carefully monitor the water bath temperature. **Higher temperatures can degrade components of the FBS.**

Note: The water level in the water bath should cover the level of the FBS in the bottle, but not touch the cap of the bottle. This will help ensure even heating of the FBS and avoid contamination.

2.5 Once the water bath has reached 56°C (55–57°C), heat the FBS for 30 minutes, mixing every 5-10 minutes. **Heating for longer periods of time can degrade components of the FBS.**

2.6 Mix the FBS gently but thoroughly and using aseptic technique, aliquot into sterile, labeled 50mL conical tubes. *Note*: Labels should identify these tubes as "HI-FBS" and include the lot number, the aliquot date, the expiration date, and your initials.

Note: FBS is table for 1 month at 2-8°C, or the original manufacturer's expiration date at 20°C.

2.7 Refrigerate (2°-8°C) the number of aliquot tubes you need for your expected monthly workload. Mix well before use. Refrigerated FBS is stable for one month. *Note*: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

2.8 The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacture's expiration date.

2.9 When ready to use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well

before use.

3. Fresh Cryopreservation Solution (CPS)

3.1

Components	Percent	Example
DMSO	10%	5mL
HI-FBS (heat-inactivated)	90%	45mL

3.2 Preparation of CPS: Prior to processing, use a sterile, disposable 15mL or 50mL container to prepare fresh CPS. Mixing of DMSO and FBS is an exothermic reaction, so be sure to chill the CPS in the refrigerator (2-8°C) or in an ice bath for at least 30 minutes prior to use. Store at 28 °C for up to 1 week. Alternatively, larger volumes can be prepared, filter-sterilized*, aliquoted and stored at –20 °C for up to one year. **Note:* DMSO may damage the filter. Do NOT filter DMSO directly. Mix DMSO with the FBS prior to filtration to preserve the integrity of the filter.

3.3 Use the formula below to estimate the volume of CPS needed.

Examples: Adult Blood – Large Volume Blood Collection

Usable Whole Blood x	Average Cell Yield x	Freeze-down Concentration =	Estimated CPS
(85mL) x	(1.5x106 cells/1mL) x	(1mL/15x106 cells) =	~9mL
(140mL) x	(1.5x106 cells/1mL) x	(1mL/15x106 cells) =	14mL

Examples: Adolescent/Pediatric Blood – Small Volume Blood Collection

Usable Whole Blood x	Average Cell Yield x	Freeze-down Concentration =	Estimated CPS
(10mL) x	(1.5x106 cells/1mL) x	(0.5mL/5x106 cells) =	1.5mL
(2mL) x	(5x106 cells/1mL) x	(0.5mL/2.5x106 cells) =	2mL

Use the following formulas to calculate the amount of DMSO and FBS needed. Examples:

Estimated CPS Volume	DMSO Volume = **(.1)(CPS volume)**	HI-FBS Volume = **CPS volume – DMSO volume**	Total CPS Volume = **DMSO volume + FBS volume**
1mL	0.1mL	0.9mL	1mL
9mL	0.9mL	8.1mL	9mL
50mL	5mL	45mL	50mL

Record CPS, DMSO and HI-FBS volumes.

4. Wash Solution (Merck Only)

4.1 Prepare HBSS + 5% FBS (e.g. 25mL FBS +475mL HBSS). Store refrigerated (2-8^{o}C) for up to 1 month.

Counting Reagents

The requirements for counting reagents will vary depending on the method used. See the instructions for the method you will be using for the necessary cell counting reagent.

Calibration

1 **No calibration is required for the processing steps.**
2 **Follow the applicable laboratory calibration procedures if using an automated cell counter.**

Quality Control

Cell yields are fairly consistent within populations. Infant populations typically generate higher lymphocyte yields than adult populations. Similarly, patients with AIDS or advanced HIV disease may be lymphopenic. It is important to be aware of the expected recovery that should be obtained for the population of patients for which the processing is being done. Yields outside the expected ranges may indicate a procedural error, reagent deterioration, cell count error, or calculation error. The recommendations provided below are meant to provide a guideline to help identify egregious technical errors prior to cryopreservation.

Expected Cell Yields

2.1 Adult population: In most adult populations, the expected yield of PBMC is approximately 1.5x10^{6} cells/mL with a range of 0.8 – 3.0 x10^{6} cells/mL of usable blood.

2.2 Pediatric population: Expected pediatric cell count/mL differs by age range: newborns (less than 6 months) 3-10x10^{6} cells/mL; 6 mo. to 2 years 2-6x10^{6} cells/mL; 2-5 years 1-4x10^{6} cells/mL; more than 5 years 0.8-3.0 x10^{6} cells/mL. If the age is unknown to the processing lab use the full range of 1-10x10^{6} cells/mL. If pediatric cell yields are below 1x10^{6} or above 10x10^{6} cells/mL of usable blood make a fresh dilution and re-count to rule out dilution or counting error.

Unexpected Cell Yields

2.3 If the cell yield is outside 1-3 x10^{6} cells/mL of usable blood for adult specimens or 1-10x10^{6} for pediatric specimens, make a fresh dilution and recount to rule out a dilution or counting error.

2.4 If the cell recount is consistent with the original cell count, record both cell counts on the PBMC Processing Worksheet and continue processing.

2.5 If the recount is inconsistent with the original cell count, make another fresh dilution and recount a third time. If two of the three counts fall within your laboratory's acceptable precision limits, use the average of the two closest results for your calculations. If the imprecision persists, consult your supervisor.

2.6 Record all results and any problems that occur during processing on the PBMC Processing Worksheet.

PBMC Worksheet

2.7 The use of a PBMC processing worksheet is strongly recommended to track the timing of processing, calculations and documentation of problems that arise during processing. An example of a PBMC worksheet may be viewed in the appendices.

PBMC Processing

Note: Although, the following describes procedures using Accuspin tubes, other cell separation tubes with frit barriers may be used.

1. Separation of lymphocytes from peripheral blood using Accuspin separation tubes (See Appendix 2 for Ficoll Hypaque Overlay Method)

1.1 All pipetting and mixing takes place in a biological safety cabinet (BSC), level 2 or greater.
1.2 Spray down all surfaces, racks, and reagent bottles with disinfectant prior to entering and using the BSC Hood.
1.3 Unless otherwise noted, the procedure is carried out at room temperature (15-30°C).
1.4 Use a new pipet for each participant identification number (PTID).

2. Prepare whole blood samples, reagents, and supplies.

2.1 Prior to processing, prepare and chill the CPS.
2.2 Allow the Accuspin tubes to come to room temperature (15-30°C). See the Reagent section of this document for more information.
2.3 Record on the worksheet: the PTID, visit number, protocol, collection date/time, processing start-date and time, lot numbers and expiration dates of all reagents, CPS, DMSO, and FBS volumes.
2.4 Before adding the blood, visually check the Accuspin tubes to see if there is liquid above the frit. If there is liquid above the frit, centrifuge the Accuspin tubes at 1000 x g for 30 seconds.
2.5 Carefully check the PTID on all tubes of blood received.
2.6 Place all tubes for each PTID in a rack. If possible, do not mix two PTIDs or tube types in the same rack.
2.7 If the specimen tubes are cold to the touch (due to cold ambient conditions such as transport in cooler months), allow the tubes to reach room temperature (15-30°C) before processing.
2.8 Label each Accuspin tube with the PTID. You will need 1 tube for each 15-20mL of whole blood.
2.9 Uncap the tubes of anti-coagulated blood.
2.10 If a tube is grossly clotted (see glossary), discard it.
2.11 Document the type of specimen received.
2.12 Record the total number of tubes discarded.

2.13 Record the blood condition.

3. Blood Dilution for Accuspin separation

Note 1: An accurate measurement of the usable blood volume must be determined and recorded on the PBMC worksheet. This can be accomplished by using a sterile pipette to transfer the whole blood to the Accuspin tubes and keeping track of the blood volume as it is pipetted, or by pooling the blood in a sterile graduated container.

Note 2: Dilution of blood with HBSS can help improve separation. Blood to HBSS ratios generally range from 15 mL blood: 15 mL HBSS to 20mL blood:10mL HBSS. The final combined volume above the frit in each Accuspin tube should not exceed 30mL. Use as many Accuspin tubes as required to distribute all of the blood for each PTID.

3.1 Label all tubes with PTID identifiers.

3.2 If plasma is to be removed prior to PBMC separation, spin the blood at 400 x g for 10 minutes, transfer plasma to a 15 or 50mL centrifuge tube for clarification of cellular debris (spin at 800 x g for 10 minutes), and add sufficient quantity of PBS or HBSS without calcium or magnesium to bring the blood to its original volume. Subsequent dilution of blood with HBSS (see note 2) should be performed after the blood is returned to its original volume.

3.3 Using a sterile pipette, add 5mL of HBSS to each Accuspin tube.

3.4 Using a sterile pipet, transfer 15-20mL of blood into an appropriately labeled Accuspin tube.

3.5 Using a sterile pipette, rinse each original anti-coagulated blood tube with HBSS, add rinse volumes to balance the Accuspin tubes making sure not to exceed the 30mL total tube volume (HBSS + Whole Blood). Note: blood may be diluted in HBSS prior to adding to prepared Accuspin tubes.

3.6 Record the total volume of undiluted whole blood.

Note: Do not estimate the volume of usable blood based on the tube size.

Note: Do not include the volume of HBSS used to dilute the blood sample.

3.7 Carefully cap the tubes.

4. Accuspin density centrifugation and collection

4.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

4.2 Centrifuge at 800 x g for 15 minutes at 18-26°C with the Brake OFF.
Note: If the brake is on, it will disrupt the layers. Refer to the Calculations section of this document to convert g to rpm for your rotor length.

4.3 Prepare the same number of new sterile 50mL conical tubes as Accuspin tubes used in the separation centrifugation step.

4.4 Label each tube with the PTID. Use these new tubes for the next wash.

4.5 Remove the Accuspin tubes from the centrifuge.

4.6 Inspect the tubes for hemolysis or small clots visible on the Accuspin frit that had not previously been noted and document.

4.7 Re-evaluate the blood condition.

Note: Look for hemolysis, or clots after centrifugation. Grade hemolysis +1 through +4 based on the description given in the glossary. Record your observations.

4.8 Using a new sterile pipette (serological or transfer pipette) for each PTID, remove the upper, yellowish, plasma-HBSS fraction down to within approximately 2 cm (5mL) of the cloudy-white PBMC band located at the interface between the plasma-HBSS (yellowish) fraction and the clear separation medium solution. Discard the plasma-HBSS fraction per laboratory policy.

4.9 Using a sterile serological or transfer pipette, collect all cells at the cloudy-white interface above the frit. Use the tip of the pipette to gently scratch the inner walls of the tube at the interface to collect any cells stuck to the tube. Take care not to aspirate any more separation medium solution than necessary.

Note: Alternatively for steps 4.8 and 4.9, the upper plasma-HBSS fraction may be left in place and the cloudy-white PBMC band may be removed by carefully inserting the pipette through the upper layer to the PBMC band.

4.10 Transfer the collected cells from one Accuspin tube to a single corresponding, pre-labeled, sterile 50mL conical tube.

4.11 Re-cap the Accuspin tube containing the remaining red blood cells and separation media.

4.12 Discard the Accuspin tube as biohazard waste following laboratory policy.

5. Wash 1:

5.1 Using a sterile 50mL pipette, add HBSS to each tube containing the collected cells to bring the final volume up to 45mL.

5.2 Re-cap all of the harvested cell tubes.

5.3 Centrifuge the diluted cells at 250 x g for 10 minutes at 18-26°C with the brake on

5.4 Remove the tubes from the centrifuge and check for the cell pellet.

5.5 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-spin the tube. Document the problem and actions taken.

If the cell pellet is still not visible after re-spinning the tube, document, remove and discard the HBSS supernatant and proceed.

6. Wash 2:

6.1 Note: For Merck protocols use HBSS+5%FBS.

6.2 Re-suspend the pellet in a small volume (≤5mL) of HBSS mixing gently, but thoroughly, into a homogenous cell suspension.

6.3 Combine up to four pellet suspensions from the same donor into one 50mL conical tube.

This is your harvested cell tube.

6.4 Use a small volume of HBSS to rinse the tubes from which the pellets were transferred. *Note:* The combined rinse and harvested volume should not exceed 45mL.

6.5 Collect the HBSS rinse in the harvested cell tube.

6.6 Re-cap the tubes and place the tubes in the centrifuge.

6.7 Centrifuge at 250 x g for ten minutes at 18-26°C with the brake on.

6.8 Remove the tubes from the centrifuge and check for the cell pellet.

6.9 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Respin the tube. Document the problem and actions taken.

If the cell pellet is still not visible after respinning the tube, document, remove and discard the HBSS supernatant. Keep the pellet.

7. Merck ACK Lysing Step (Merck protocols only)

7.1 Add ACK lysing buffer as follows:

7.2 3mL of ACK to each cell pellet pooled from < 50 mL blood sample

7.3 6mL of ACK to each cell pellet pooled from ≥ 50 mL blood sample

7.4 Let sit for 3-5 minutes.

7.5 Bring volume in tube up to 45 mL with HBSS+5%FBS. Centrifuge at 250 x g for 10 minutes at 20±2 ºC.

7.6 Discard supernatant and re-suspend cell pellet in 20 mL of HBSS+5%FBS. Centrifuge at 250 x g for 10 minutes at 20±2 ºC.

8. PBMC Cell Count

8.1 Record the Counting method used on the PBMC Processing Worksheet,

8.2 Calculate and record on the processing worksheet, the HBSS counting re-suspension volume (V). This is the volume on which the cell count is based.

Note: The re-suspension volume should be approximately 20% of the usable whole blood volume rounded to the nearest mL.

8.3 If there is more than one pellet, use ½ of the counting re-suspension volume of HBSS to gently re-suspend and combine the cell pellets into one tube.

8.4 Using the remaining volume, rinse the tubes from which the cells were transferred.

8.5 Add the rinse to the harvested cell tube.

8.6 Complete the cell count using the SOP for the cell counting method approved at your laboratory.

8.7 Mix cells gently, but thoroughly, before sampling for your count.

8.8 Transfer a small volume of the re-suspension to a small tube for counting.

Note: If repeated counts are necessary, minimize the sampling volume needed.

8.9 Follow the SOP for the cell counting method approved at your laboratory and the

PBMC Processing Worksheet to determine the cell concentration x 10^6 per mL.

Note: Cells at 10^3/µL = cells at 10^6/mL.

8.10 Using the appropriate Cell Counts section for your method on the back of the PBMC Processing Worksheet, record the cell count concentrations for each PTID (cells x 10^6 per mL).

Note: Automated counts may be run once. Manual counts should count at least the four large corner squares ($1mm^2$).

8.11 On the front of the PBMC Processing Worksheet, record the automated or manual cell count.

8.12 Calculate the total number of cells using the following formula:

T = C x V T = Total number of cells C = Concentration (10^6/mL) determined in counting method V = Count re-suspension volume of HBSS in mL

8.13 Record the total number of cells (T) on the PBMC Processing Worksheet.

8.14 Calculate the cell yield in cells/mL of usable whole blood using the formula below.

Cell Yield (10^6 cells/mL) = T /Usable Whole Blood Volume

8.15 Record the cell yield on the PBMC Processing Worksheet.

Note: The cell yield is calculated for quality purposes only. Refer to the Quality Control section of this document for the expected range of cell yields. If the cell yield is outside of the expected range, re-dilute and re-count following Quality Control guidelines.

8.16 Record any anomalies in processing.

9. Quality Control:

9.1 If the cell yield is outside the expected range make a fresh dilution and re-count to rule out dilution or counting error.

9.2 If the cell recount is consistent with the original cell count, record both cell counts on the PBMC Processing Worksheet and continue processing.

9.3 If the recount is inconsistent with the original cell count, make another fresh dilution and recount a third time. If two of the three counts fall within your laboratory's acceptable precision limits, use the average of the two closest results for your calculations. If the imprecision persists, consult your supervisor.

9.4 Record all results and any problems that occur during processing on the PBMC Processing Worksheet.

10. Final re-suspension volume and concentration for freeze-down

10.1 Note: For Merck, use the "Table for Cell Freezing" to determine the re-suspension volume.

10.2 Calculate the CPS freeze-down re-suspension volume required by completing the steps below for a desired final cell concentration range between:

HVTN: 15 - 18 x 10^6 cells/mL (15-18 x 10^6 cells/vial)
ACTG: 10 x 10^6 cells/mL (5-10 x 10^6 cells/vial)
PACTG/IMPAACT: 5 - 10 x 10^6 cells/mL (2.5 - 5 x 10^6 cells/vial)

Note: The following example is based on a desired cell concentration of 15 x 10^6 cells/mL. Adjust the calculation as needed for different Network requirements.

Calculate the estimated CPS freeze-down re-suspension volume (V1) required by using a desired final cell concentration of 15 x 10^6 cells/mL at one mL per vial.

V1 = (T/N1) x V2

T = Total number of cells

N1 = Desired number 15 x 10^6 cells per vial

V2 = 1mL per vial

Record the estimated volume (V1) on the PBMC Processing Worksheet. Round V1 down to the nearest whole mL to determine the actual CPS re-suspension Volume (Vf). The acceptable range of cells/vial is 10-20 x 10^6 cells. *Note:* PBMC aliquot volume = 1mL/vial. The milliliters of CPS will equal the number of vials required. Record the Actual CPS re-suspension volume (Vf) on the PBMC Processing Worksheet.

10.3 Calculate the actual number of cells per vial (N2) using the actual CPS freeze-down volume (Vf) determined in the previous calculation.

N2 = (T/ *Vf*) x V2

N2 = Actual number of cell per vial

T = Total number of cells

V2= 1 mL per vial

10.4 Record the final number of cells per vial (N2) on the PBMC Processing Worksheet.

10.5 Confirm that the PBMC Processing Worksheet is complete and that the calculations are correct.

11. Labeling

11.1 Complete the printing and labeling of the cryovials PRIOR to the final centrifugation. *Note:* This is important to ensure that cells do not sit in a pellet for an extended period of time.

11.2 Apply the labels on the cryovials so that the information can be easily read and the contents of the tube can be clearly seen.

12. Final Centrifugation

12.1 Place the harvested cell tube in the centrifuge.

12.2 Centrifuge the cells at 250 x g for 10 minutes at 18-26°C with the brake on.

12.3 Verify that all cryovials are labeled and available in your workspace.

13. Aliquoting for cryopreservation

Note: The following steps should be performed quickly to preserve cell integrity. It is permissible, although not necessary, to work on wet ice.

13.1 Remove and discard the HBSS supernatant. Keep the pellet.

13.2 Re-suspend the pellet using the calculated volume of cold CPS (Vf).

13.3 Work quickly once the CPS has been added.

13.4 Aliquot 1 mL per tube (unless protocol specifies different final volume); evenly distribute any excess volume (due to the cell pellet size) among all of the tubes for that PTID.

14. Overnight controlled-rate freezing

14.1 Select the freezing method available in your laboratory.

Follow the manufacturer's guidelines for use of StrataCooler or Mr. Frosty. *Note*: Before transferring cryovials to the StrataCooler or Mr. Frosty, make sure the containers have reached the appropriate temperature (StrataCooler = 2-8ºC, Mr. Frosty = room temperature)

Follow the appropriate on-site SOP for Cryomed.

14.2 Immediately transfer all cryovials to the controlled-rate freezing container.

For Mr. Frosty and StrataCooler, close the container and place it in a –70/-80°C freezer for at
least 24 hours.
For Cryomed, start the cooling program.

14.3 Record the date and time the rate controlled freezing was initiated on the PBMC Processing Worksheet.

Note: This is the completion time of processing.

14.4 Record the actual number of cryovials frozen on the PBMC Processing Worksheet.

15. Transfer of PBMC cryovials at –70/-80ºC (HVTN, Merck or other networks using –70/-80ºC as temporary storage for dry ice shipments)

15.1 Transfer the cryovials from the controlled-rate cooling system to the designated storage location at -70/-80 °C. Note: Do not store in liquid nitrogen.

For StrataCooler or Mr. Frosty, transfer the cryovials after 24 hours at –70/-80°C. *Note:* Allow StrataCooler to equilibrate to 2-8°C, and Mr. Frosty to equilibrate to room temperature prior to next use.

If you are using Cryomed, transfer the cryovials upon completion of the program to the –70/80°C freezer.

15.2 Record the initials of the person making the transfer and the date/time of the transfer.

15.3 Record the appropriate storage information based on your laboratory's defined storage program.

16. Transfer of PBMC cryovials to Liquid Nitrogen (refer to network/protocol specifications prior to moving PBMC to LN2)

16.1 Transfer the cryovials from the controlled-rate cooling system to the designated storage location in the liquid nitrogen storage system.

16.2 Record the initials of the person making the transfer along with the date/time of the transfer on the PBMC processing worksheet.

16.3 Record the appropriate storage information on the PBMC Processing Worksheet based on your laboratory's defined storage program.

17. Final Worksheet Review

17.1 The technician should confirm that the PBMC Processing Worksheet is complete and that the calculations are correct.

17.2 A second reviewer checks the worksheet for completeness and accuracy and then initials and dates the PBMC Processing Worksheet. *Note*: All reviews should occur within two days of processing.

17.3 Store the PBMC Processing Worksheet according to laboratory policy.

18. Storage Guidelines

18.1 Short Term Freezer Storage pending dry ice shipment

Store between -65°C to -94°C until shipped.

Acceptable temperature range for short-term storage of PBMC is -64°C to -94°C for up to three weeks from the date of freezing.

Dry-ice shipments must be shipped to the central specimen repository within two weeks from the date of freezing. *Note:* Samples for Merck protocols should be shipped weekly.

Once samples have been stored on dry ice, all transfers or shipments must be maintained on dry ice. Never transfer samples from dry ice back to -70 to -80°C freezers.

Contact network laboratory operations personnel if samples cannot reach their final destination within 21 days from the date of freezing. Permission to move samples to liquid nitrogen storage and ship in liquid nitrogen shippers is needed if the 21 day deadline cannot be met.

18.2 Long-term liquid nitrogen storage

Frozen PBMC samples can be stored safely in liquid nitrogen indefinitely (vapor phase preferred).

Once samples have been stored in liquid nitrogen, all transfers or shipments must be

maintained in liquid nitrogen (≤ –140°C).

Once samples have been stored in liquid nitrogen, samples cannot be shipped on dry ice.

Do not temporarily store samples in liquid nitrogen.

Never transfer samples from liquid nitrogen back to -70°C or -80°C freezers.

Once samples are stored in liquid nitrogen, the samples must remain in liquid nitrogen

until

thawed for use, including during shipping.

This marks the end of processing and storage. Follow the appropriate laboratory procedures for preparation and processing of shipments.

Reporting Results (if applicable)

1 Frozen PBMC is reported as PBMC cells per 1mL in CPS.
2 Original results are reported on the PBMC Processing Worksheet.
3 Upon completion of processing, the processor verifies that the PBMC Processing Worksheet is complete and that the calculations are correct.
4 A second review should catch and correct processing errors before specimens leave the site. This review should take place within 2 days of the PBMC processing.
5 After the second review, the PBMC Processing Worksheet may be faxed to the appropriate network contact for final review.
6 Cryovials should be labeled according to the specifications of the network for which the original processing was performed. Where appropriate, data should be entered into the LDMS to permit proper labeling, tracking and shipping of specimens.
7 Deviations and amendments are reported according to laboratory protocol.

Calculations

1 The RPMs are usually read off a nomogram chart. Nomogram charts are often included in the centrifuge maintenance manual. Be sure to use centrifuge and rotor specific charts.
2 It is recommended that you post the g to RPM conversion on your centrifuge for easy reference.
3 If you are unable to find a nomogram chart, g's can be converted to RPM's using the following formula.

$$RPM^2 = g/(1.18 \times 10^{-5})(r)$$

r = radius of rotor

g = relative centrifugal force expressed in units of gravity

RPM = revolutions per minute

Limitations of the Procedure

1 Optimum processing time is <8 hours from the time of collection. Cell function may drop for older specimens.
2 Avoid removing excess amounts of the separation media with the PBMC band as that can increase granulocyte contamination.

3 Avoid removing excess supernatant with the PBMC band to limit contamination from plasma proteins.

Procedural Notes

1 If the plasma is very cloudy, it may be difficult to see the interface of the Ficoll gradient. It is possible to improve the collection of lymphocytes by removing most of the plasma above the interface with a 10mL pipet, leaving only 3-4mL remaining. This allows for better positioning of the tip of the pipet for collection of cells.
2 Liquid nitrogen vapor-phase storage is the space in the storage tank that is above the LN2 liquid at the bottom of the tank.
3 Modification of the centrifuge speed to 250 x g is based on the information provided by the Sigma-Aldrich Accuspin System-Histopaque-1077 package insert, dated 2003/09.

Glossary of Terms References

Term	Definition
PTID	Participant Identification Number
Centrifuge Temperature	18-26oC
CPS	Cryopreservation Solution
CSR	Central Specimen Repository
CSTFB	Cell separation tube with frit barrier (such as Accuspin, Leucosep or Lymphoprep)
FBS	Fetal Bovine Serum
Grossly Clotted	More than ¼ of the whole blood mass is clotted and there is very little free whole blood remaining.
HBSS	Hanks' Balanced Salt Solution
Hemolysis	A pink to red coloration of serum or plasma due to the lysis of red blood cells. Hemolysis is graded and reported according to the following scale: 1+ Pale pinkish-red color in serum or plasma 2+ Pinkish-red color in serum or plasma 3+ Dark pinkish-red color in serum or plasma 4+ Dark red mahogany color in serum or plasma
HI-FBS	Heat Inactivated Fetal Bovine Serum
Icteric	A green or orange tinted plasma suggesting the presence of increased bilirubin.
LDMS	Laboratory Data Management System

PBMC	Peripheral Blood Mononuclear Cells

Term	**Definition**
Room Temperature	15-30oC
Small Clots	Small clots will not usually be seen in the whole blood tube, but can be seen on the Accuspin tube frit after centrifugation.

1 Boyum A. Isolation of mononuclear cells and granulocytes from human blood, Scand. J. Clin. Lab. Invest. 21:77-89 (1968).
2 Islam D., Lindbert A., and Christensen B. Peripheral blood cells preparation influences the level of expression of leukocyte cell surface markers as assessed with quantitative multicolor flow cytometry. Cytometry 22:128-134 (1995).
3 Weinberg A., Betensky R., Zhang L., and Ray G. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus- infected patients. Clin. Diagn. Lab. Immunol. 5:804-807 (1998).
4 Cox J.H., DeSouza M., Ratto-Kim S., Ferrari G., Weinhold K.J., and Birx D.L. Cellular Immune assays for evaluation of Vaccine efficacy in developing countries. Manual of Clinical laboratory Immunology. Rose N.R., Hamilton R.G., Detrick B. Eds. (6th ed.) p.301-315 (2002).
5 Sigma-Aldrich Accuspin System-Histopaque-1077, procedure number A6929/A7054/A0561, Dated 2003-09.

Appendices

1 Sample PBMC Processing Worksheet (Non-Merck)
2 Ficoll Hypaque Overlay Method

Additional Documents (To be Maintained by the Laboratory)

1 FBS Package Insert (and Certificate of Analysis)
2 HBSS Package Insert
3 Density Gradient Medium Package Insert
4 Cell separation tube with frit barrier package insert

Appendix 1: Appendix 2:

PBMC Processing Worksheet

PTID_____ Visit_____ Protocol:_____

Collection Date_____ Time_____

Processing Start Date_____ Time_____

Processed By_____

Reagents	Lot Number	Expiration Date
DMSO		
HI-FBS		
HBSS		
Separation Tubes/Media Accuspin/Leucosep (circle one) DG Media_____		

	Volume in mls			
	CPS	DMSO	FBS	
CPS				8 hours

Data to be Captured During Processing	Sample
Sample tube type (circle one)	NaHep ACD _____
Blood condition (circle one or more)	NORM / HEMO/ CLOTTED
Usable whole blood volume	ml
Counting Method (name of instrument or manual count)	
Counting re-suspension volume of HBSS (V)	ml
Cell count average concentration (C)	x 10^6 cells/ml
Total cell number (**T**) = V x C	x 10^6 cells
Calculate cell yield/ml of whole blood (QC check)= *(T/Usable Whole Blood Volume)*	x 10^6 cells/ml
Calculate estimated CPS re-suspension vol. ***V1***=*(T/15 x10^6 cells)(1ml)*	ml
Calculate the final CPS re-suspension volume (V_f), rounded DOWN to the nearest whole ml	ml
Calculate actual number of cells per vial Optimum range: 15.0-18.0 x 10^6 , Acceptable limit: 10-20 x 10^6 ***N2*** = *(T)/(V_f))*	x 10^6 cells/vial
Date and time processing completed - (Note in comments if not within 8 hours of processing start time)	

Participant ID number_____

Number of cryovials actually frozen Note: Should be equal to freeze down re suspension volume	
Transfer	
Person transferring cryovials to storage box locations assigned by LDMS	
Date (ddmmmyy)/time cryovials were transferred from slow-rate cooling device to storage box. (Samples must be maintained at -70 or -80°C during transfer)	
Final Review	
Reviewer/Date	

Hemacytometer Counts	**Original count**	**Recount #1**	**Recount #2**	**Average**
Square #1 (cells/mm^2)				
Square #2 (cells/mm^2)				
Square #3 (cells/mm^2)				
Square #4 (cells/mm^2)				
Average Cell Count per Square (cells/mm^2)				
PBMC Dilution Factor (1: DF*)				
Hemacytometer Factor for cells/ml	10^4	10^4	10^4	10^4
Cell count concentration (C) = (Average Cells/mm^2)(DF)(10^4); convert to 10^6 cells/ml	x 10^6cells/ml	x 10^6cells/ml	x 10^6cells/ml	x 10^6cells/ml
% viability = (viable cells/total cells)(100)				

Automated Cell Counts (10^3/µl=10^6/ml)	**Original Cell Count**	**Recount #1**	**Recount #2**	**Average**
Cell Count (C) as cells x 10^6/ml				
PBMC Dilution Factor (1: DF*)				
Cell Concentration= (C)(DF)	x 10^6cells/ml	x 10^6cells/ml	x 10^6cells/ml	x 10^6cells/ml

*Note: Dilution Factor (DF) = (parts cells + parts dilution fluid)/ parts cells

Guava Counter Cell Counts (10^3/µl=10^6/ml)	**Original Cell Count**	**Recount #1**	**Recount #2**	**Average**
Cell Count (C) as cells x 10^6/ml				
Total Cells (T) as cells x 10^6				
% viability				

Comments:

PBMC Processing (Ficoll Hypaque Overlay Method)

1. Separation of lymphocytes from peripheral blood using Ficoll Hypaque Overlay Method

1.1 All pipetting and mixing takes place in a biological safety cabinet, level 2 or greater.
1.2 Spray down all surfaces, racks, and reagent bottles with 70% ethanol prior to entering and using the BSC Hood.
1.3 Unless otherwise noted, the procedure is carried out at room temperature (15-30°C).
1.4 Use a new pipet for each participant identification number (PTID).

2. Prepare whole blood samples, reagents, and supplies (utilize the PBMC Processing Worksheet or other tracking tool as defined by the protocol or network to document and track specimen processing).

2.1 Prior to processing, prepare and chill the CPS.
2.2 Record the PTID, visit number, protocol, and collection date/time.
2.3 Record the processing start date/time
2.4 Record the lot numbers and expiration dates of all reagents.
2.5 Carefully check the PTID on all tubes of blood received.
2.6 If the specimen tubes are cold to the touch (due to cold ambient conditions such as transport in cooler months), allow the tubes to reach room temperature (15-30°C) before processing.
2.7 Label each 15 or 50mL centrifuge tube with the PTID. You will need one 50mL tube for each 15-20mL of adult whole blood (or one 15mL tube for each 5mL of pediatric whole blood).
2.8 Uncap the tubes of anti-coagulated blood.
2.9 If a tube is grossly clotted (see glossary), discard it.
2.10 Document the type of blood received.
2.11 Record the total number of tubes discarded or any other problems noted with the blood.
2.12 Record the blood condition.

- **Blood Dilution for Accuspin separation**

Note 1: An accurate measurement of the usable blood volume must be determined and recorded. *Note 2:* Dilution of blood with HBSS can help improve separation.

3.1 Label all tubes with PTID identifiers.

3.2 If plasma is to be removed prior to PBMC separation, spin the blood at 400 x g for 10 minutes, transfer plasma to a 15 or 50mL centrifuge tube for clarification of cellular debris (spin at 800 x g for 10 minutes), and add sufficient quantity of PBS or HBSS without calcium or magnesium to bring the blood to its original volume. Subsequent dilution of blood with HBSS (see note 2) should be performed after the blood is returned to its original volume.

3.3 Separate the peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation. Follow manufacturer's instructions with respect to blood/ ratios and centrifugation time and speed. Centrifuge at room temperature with NO brake. (The PBMCs can then be resuspended, counted and used for culture, stored as dry cell pellets, or stored as viable cell suspensions.)

3.4 If plasma was removed for storage, add a volume of sterile 1X PBS or HBSS equal to the volume of plasma removed. Mix gently and thoroughly. This step will decrease clumping of the cells during separation. Transfer the blood to a sterile, labeled 15 or 50mL

centrifuge tube and add sufficient volume of HBSS or 1X PBS to dilute the blood according to the lymphocyte separation medium package insert (minimum dilution should be 1:2 of original blood volume). For maximum yield, rinse the original blood containers with PBS or HBSS.

3.5 Record the total volume of undiluted whole blood.

Note: Do not estimate the volume of usable blood based on the tube size.

Note: Do not include the volume of HBSS used to dilute the blood sample.

3.6 Add density gradient medium to a clean, sterile, labeled 15 or 50mL centrifuge tube. **Note**: the ratio of gradient medium to whole blood may vary according to manufacturer's recommendations and laboratory experience. For example, some manufacturers recommend 4 parts diluted blood to 3 parts gradient medium; however practical experience has shown good results using 3 parts blood to 1 part gradient medium.

3.7 Carefully and slowly pipet diluted blood on top of gradient medium. Suggestion: gently allow mixture to flow down side of tube and pool on top of ficoll surface w/o breaking surface plane.

3.8 Carefully cap the tubes.

4. Lymphocyte density centrifugation and collection

4.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

4.2 Centrifuge at 300 - 800 x g for 15 -30 minutes at 18-26°C with the Brake OFF as outlined in the package insert that accompanies the gradient medium.

Note: If the brake is on, it will disrupt the layers. Refer to the Calculations section of this document to convert g to rpm for your rotor length.

4.3 Prepare the same number of new sterile 15mL or 50mL conical tubes as centrifuge tubes used in the separation centrifugation step.

4.4 Label each tube with the PTID. Use these new tubes for the next wash.

4.5 Remove the tubes from the centrifuge.

4.6 Inspect the tubes for hemolysis or small clots visible at the cell interface that were not previously noted and document them.

4.7 Re-evaluate the blood condition.

Note: Look for hemolysis, or clots after centrifugation. Grade hemolysis +1 through +4 based on the description given in the glossary. Record your observations.

4.8 Using a new sterile pipette (serological or transfer pipette) for each PTID, remove the upper, yellowish, plasma-HBSS fraction down to within approximately 2 cm (5mL) of the cloudy-white PBMC band located at the interface between the plasma-HBSS (yellowish) fraction and the clear separation medium solution. Discard the plasma-HBSS fraction per laboratory policy.

4.9 Using a sterile serological or transfer pipette, collect all cells at the cloudy-white interface. Use the tip of the pipette to gently scratch the inner walls of the tube at the interface to

collect any cells stuck to the tube. Take care not to aspirate any more separation medium solution than necessary.
Note: Alternatively for steps 4.8 and 4.9, the upper plasma-HBSS fraction may be left in place and the cloudy-white PBMC band may be removed by carefully inserting the pipette through the upper layer to the PBMC band.

4.10 Transfer the collected cells from one centrifuge tube to a single corresponding, pre-labeled, sterile 15 or 50mL conical tube.

4.11 Re-cap the centrifuge tube containing the remaining red blood cells and separation medium.

4.12 Discard the Accuspin tube as biohazard waste following laboratory policy.

5. Wash 1:

5.1 Using a sterile 50mL pipette, add HBSS or PBS (Note: For Merck protocols use HBSS+5%FBS) to each tube containing the collected cells to bring the final volume up to 45mL.

5.2 Re-cap all of the harvested cell tubes.

5.3 Centrifuge the diluted cells at 250 x g for 10 minutes at 18-26°C with the brake on

5.4 Remove the tubes from the centrifuge and check for the cell pellet.

5.5 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-spin the tube. Document the problem and actions taken.

If the cell pellet is still not visible after re-spinning the tube, document, remove and discard the HBSS supernatant. Keep the pellet.

6. Wash 2:

6.1 Re-suspend the pellet in a small volume (≤5mL) of HBSS or PBS (Note: For Merck protocols use HBSS+5%FBS) mixing gently, but thoroughly, into a homogenous cell suspension.

6.2 Combine up to four pellet suspensions from the same donor into one 50mL conical tube. This is your harvested cell tube.

6.3 Use a small volume of HBSS to rinse the tubes from which the pellets were transferred. *Note:* The combined rinse and harvested volume should not exceed 45mL.

6.4 Collect the HBSS rinse in the harvested cell tube.

6.5 Re-cap the tubes and place the tubes in the centrifuge.

6.6 Centrifuge at 250 x g for ten minutes at 18-26°C with the brake on.

6.7 Remove the tubes from the centrifuge and check for the cell pellet.

6.8 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Respin the tube. Document the problem and actions taken.

If the cell pellet is still not visible after respinning the tube, document, remove and discard the HBSS supernatant and proceed.

Owners, Name&Title	Constance Ducar, International Laboratory Program Manager Donna Smith, International Laboratory Program QA Associate

Approved By	Name, Title	signature	date
	Constance Ducar International Lab Program Mgr		
	John Hural Lab Operations Associate Director		

Revision History	Version	Effective Date [ddmmmyy]	Description (notes)
	1.0	03Aug05	Rewrite of SOP #LP-001, revision of wash steps
	2.0	15Jun06	Update QC section. Harmonize w/CHAVI & Cross-Network PBMC by adding: Optional plasma replacement step, optional CST centrifuge time, CPS-cell time warning, revised worksheet and Appendices for PBMC workflow summary, Ficoll manual method, PBMC recovery procedure.
	3.0	24Jul06	Clarify DMSO expiration dating (4.2). Correct centrifuge time (5.2). Add DG to glossary.

SOP Annual Review	Name, Title	signature	date

Purpose

This Standard Operating Procedure (**SOP**) describes a procedure for the isolation and cryopreservation of Peripheral Blood Mononuclear Cells (**PBMC**) from whole blood.

Scope

This procedure is to be utilized for processing blood samples for the isolation, cryopreservation, and storage of PBMC samples obtained for vaccine studies conducted by the HIV Vaccine Trials Network (**HVTN**).

Background

Freshly collected or cryopreserved PBMC are used for the evaluation of vaccine or antiretroviral therapy-induced cellular immune responses, HIV-associated changes in immune response, and recovery of replication competent virus. Validation studies conducted across networks indicated that blood must be processed and frozen within 8 hours from the time of blood draw to maintain maximum function of the cells in immune-monitoring assays. These assays require PBMC that have been isolated and cryopreserved under strictly defined conditions that ensure optimal recovery, viability, and functionality.

Authority and Responsibility

1 The Associate Director (or his/her designee) of Laboratory Operations and the International Laboratory Program Manager have the authority to establish this procedure.
2 The HVTN Laboratory Program central and the local site Quality Assurance Units are responsible for the control of SOP documentation.
3 The Principal Investigator/Laboratory Manager is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.
4 All technicians working on HVTN studies are responsible for reading and understanding this SOP prior to performing the procedures described.

Specimen

1. Patient Preparation

None

2. Specimen Type

Anti-coagulated whole blood drawn in blood collection tubes

3. Optimum/Minimum Specimen Volume

Required blood volume determined by protocol

4. Handling Conditions

4.1 Fresh, anti-coagulated whole blood specimens should be stored at room temperature (15-30°C) from the time of collection until delivery to the laboratory/processing unit.
4.2 Fresh, anti-coagulated whole blood specimens should be delivered to the laboratory processing unit as soon as possible after collection and no later than 4 hours after collection to allow the processing laboratory ample time to complete the cryo-preservation procedures.
4.3 Fresh, anti-coagulated whole blood specimens should be processed by the laboratory processing unit as soon as possible upon receipt so that the rate controlled freezing is initiated within 8 hours of collection.
4.4 Do not refrigerate or freeze whole blood.
4.5 If specimens are received at temperatures below 15°C, allow whole blood to come to room temperature (15-30°C) before processing.
4.6 The use of a processing worksheet is required to track the timing of processing and document problems that may arise during processing. Record the time that processing began on the **PBMC Processing Worksheet**.
4.7 If some of the specimen blood tubes contain small clots, try to remove the clots prior to processing. Record the total number of tubes that contained small clots in the comments section on the **PBMC Processing Worksheet**.

5. Unacceptable Specimens

5.1 Unlabeled or mislabeled specimens will be rejected.

5.2 Grossly hemolyzed (4+) specimens should not be used for PBMC.

5.3 If a specimen tube is grossly clotted (see glossary), remove that tube from the specimen batch and process the remaining tubes. Record the total number of tubes that were discarded due to gross clots or gross hemolysis in the comments section on the **PBMC Processing Worksheet**.

5.4 Document all unexpected specimen conditions on the PBMC Processing Worksheet and enter the information into a laboratory data management system, such as LDMS.

Equipment

Recommended vendors and equipment are listed. Unless otherwise specified, equipment of equal or better quality than those recommended can be used.

1. Preparation & Processing

1.1 Biological laminar flow safety cabinet, as set up by laboratory (P2, P2.5 or P3)
1.2 Centrifuge, low-speed (capable of 300 to 1000 x g),refrigerated preferred, ambient acceptable
1.3 Micropipettes, range 20, 200, 1000µL
1.4 Pipette-Aid (cordless preferred) for disposable, serological pipets
1.5 2-8°C refrigerator
1.6 –20°C freezer (for FBS storage)
1.7 –70/-80°C freezer (for short term PBMC storage)
1.8 37-56°C water bath
1.9 Bleach bucket

2. Backup equipment (optional)

2.1 Liquid nitrogen storage tank (≤ –140°C)
2.2 Liquid nitrogen dry shipper

3. Cell Counting (select one of following options)

3.1 Automated cell counter (Beckman-Coulter, Abbott CelDyne, Sysmex, Guava or equivalent)
3.2 Manual cell counting chamber (Neubauer hemacytometer) with light-field microscope for manual cell counting method

4. Cryopreservation (use one of following options)

4.1 StrataCooler (Stratagene). StrataCooler must be at 2-8°C before starting the cool down of the cryovials. Do not place cryovials in StratoCooler below 2°C.

4.2 Mr. Frosty, 1^{0}C cryo-freezing container (Nalgene). Mr Frosty must be at room temperature prior to use.

Note: This container requires an explosion proof freezer for use. Alcohol should be changed every fifth freeze/thaw cycle – a log should be used to track freeze/thaw cycles and reagent changes.

4.3 Cryomed Freezing Chamber (Gordinier)

Disposables

Recommended vendors and brands are listed. Unless otherwise specified, products of equal or better quality than those recommended can be used.

1. Plastics

1.1 Serological pipets, disposable, 5, 10, 25 & 50ml, sterile, individually wrapped

1.2 Sterile precision pipet tips, 20, 100, 200, 1000 µL

1.3 15 and 50 mL disposable centrifuge tubes, sterile, conical bottom, graduated polypropylene.

1.4 Sterile bottles/flasks, disposable, 45mm neck, Polystyrene, 250ml

1.5 Cryogenic vials (cryovials), 1.8-2 mL, externally threaded screw cap with o-ring, sterile, Polypropylene only, self-standing, graduated, leak-proof, formulated for vapor-phase liquid nitrogen preservation (-190°).

1.6 Nalgene Nunc Brand Products, catalog #377267; Wheaton, catalog #985742; Fisher Scientific, catalog #05-669-57; Corning, catalog #430659 (12.7x49mm), Sarstedt, #72.694.006.

Note: Not all cryovial brands are suitable for long-term storage in liquid nitrogen. Check with HVTN or the manufacturer prior to substitution of this item.

1.7 Optional: 5ml sterile, individually wrapped plastic transfer pipettes

2. Markers

Markers for writing on processing tubes and vials should have a fine point, and contain fast drying, indelible ink. (Example: Fisher Scientific, fine line, felt tip making pen, order #S32179).

3. Labels

Cryogenic labels suitable for -70°C and LN2 temperatures.

Examples: Cryo-Tags and Cryo-Babies from Diversified Biotech, Brady B461 or B490, Shamrock freezer labels.

Personal Protective Equipment

Personal protective equipment suitable for use with Blood Borne Pathogens is required. Follow your laboratory guidelines and practices for the handling of blood products.

1 Laboratory coat
2 Eye protection
3 Latex or nitrile Gloves, non-powdered is preferred, powdered is acceptable

Reagents

Recommended vendors are listed. Unless otherwise specified, reagents of equal or better quality than those recommended can be used.

Blood Separation Reagents

1. Wash Reagents

1.1 Hanks' Balanced Salt Solution (HBSS) without calcium or magnesium, ready-touse. (preferred vendors: Gibco/BRL Life Technologies or Sigma)

Note: Store opened bottles at room temperature (15-30°C) until used or until manufacturers expiration date. Discard if visible signs of contamination, such as cloudy appearance, develop.

2. Cell Separation Tube with Frit Barrier (CSTFB)

2.1 Accuspin System-Hypaque-1077 cell separation tubes with frit (barrier), ready-to-use, 50ml tubes with 15ml Histopaque (Sigma). Storage conditions:

- Store in the refrigerator (2-8^{o}C)
- Protect from light
- Check the manufacture's expiration date.
- Tubes shipped at ambient temperature are stable for up to 3 weeks. Refrigerate upon arrival.
- A cloudy appearance indicates deterioration of the product.
- Allow Accuspin tubes to come to RT (15-30°C) prior to use.

2.2 Alternatives to pre-filled Accuspin System:

- Dry Accuspin separation tubes
- Dry Leucosep separation tubes
- Lymphoprep prefilled separation tubes, Greiner Bio-One (Axis-Shield, Norway)

2.3 Density Gradient Media Options for Dry Cell Separation Tubes

- Sigma Histopaque
- Amersham Biosciences Ficoll-hypaque
- Axis-Shield Lymphoprep media (Greiner Bio-One)

Freezing Reagents

3. Fetal Bovine Serum (FBS), heat inactivated preferred

3.1 Preferred vendor: Gemini Bioproducts (www.gembio.com), BenchMark™ FBS or equivalent grade.

3.2 *Check with HVTN prior to vendor substitution.* Not all brands of FBS are equivalent. Issues regarding quality control, toxicity, and background must be addressed before changing vendors.

3.3 Obtain a certificate of analysis from the vendor for your quality control records.

Note: A copy of the FBS certificate of analysis may be required to export (or import) PBMC aliquots between countries.

3.4 FBS stored frozen (≤ -20^{o}C) is good until the manufacturer's expiration date.

3.5 Thawed and stored at 2-8 °C, stability is one month.

4. Dimethylsulfoxide (DMSO**) (Sigma Chemical Co., catalog #D5879 or D2650)**

4.1 Store unopened bottles at room temperature. Unopened bottles are stable for an indefinite period of time.

4.2 After opening, undiluted DMSO is stable at room temperature (15-30°C) when protected from moisture, for 2 years.

4.3 Use aseptic technique when removing DMSO from the bottle to avoid possible contamination.

4.4 Discard open bottle if visible signs of contamination are noted.

5. Disinfectant

5.1 70% Ethanol Disinfectant, spray bottle

5.2 10% Bleach, bucket and spray bottle

5.3 Other disinfectant as specified by your laboratory policy

Reagent Preparation

1. Heat Inactivated FBS – HI-FBS

Heat inactivated FBS can be ordered from the manufacturer. If you receive heat inactivated FBS, follow these instructions for thawing, aliquoting and use of HI-FBS.

1.1 Remove HI-FBS from the -20°C freezer.

1.2 Thaw overnight in the refrigerator (2°-8°C), preferred, or for several hours at room temperature. Do not allow HI-FBS to sit at room temperature any longer than necessary to complete the thawing process.

1.3 Gently swirl two or three times as the HI-FBS thaws.

1.4 Once thawed, mix the HI-FBS gently but thoroughly and using aseptic technique, aliquot into sterile, labeled 50ml conical tubes.

Note: Labels should identify these tubes as "HI-FBS" and include the lot number, the aliquot date, the expiration date, and your initials. FBS is stable for 1 month at 2°-8°C or the original manufacturer's expiration date at -20.

1.5 Refrigerate (2°-8°C) the number of aliquot tubes you need for your expected workload. Mix well before use. Refrigerated FBS is stable for one month.

Note: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

1.6 The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacture's expiration date.

1.7 To use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well before use.

2. FBS Heat Inactivation Preparation

If FBS has is not heat inactivated by the manufacturer, heat inactivate using the instructions below.

2.1 Remove FBS from the -20°C freezer.

2.2 Thaw overnight in the refrigerator (2°-8°C), preferred, or for several hours at room temperature. Do not allow FBS to sit at room temperature any longer than necessary to complete the thawing process.

2.3 Gently swirl two or three times as the FBS thaws.

2.4 Place FBS in a 56°C (55– 57°C) water bath. Carefully monitor the water bath temperature. Higher temperatures can degrade components of the FBS.

Note: The water level in the water bath should cover the level of the FBS in the bottle, but not touch the cap of the bottle. This will help ensure even heating of the FBS and avoid contamination.

2.5 Once the water bath has returned to 56°C (55–57°C), heat the FBS for 30 minutes, mixing every 5-10 minutes. Heating for longer periods of time can degrade components of the FBS.

2.6 Mix the FBS gently but thoroughly and using aseptic technique, aliquot into sterile, labeled 50ml conical tubes.

Note: Labels should identify these tubes as "HI-FBS" (heat inactivated FBS) and include the lot number, the aliquot date, the expiration date, and your initials. FBS is stable for 1 month at 2°-8°C or the original manufacturer's expiration date at -20.

2.7 Refrigerate (2°-8°C) the number of aliquot tubes you need for your expected workload. Mix well before use. Refrigerated FBS is stable for one month.

Note: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

2.8 The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacture's expiration date.

2.9 When ready to use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well before use.

3. Fresh Cryopreservation Solution (CPS)

Components	Percent
DMSO	10%
FBS (heat-inactivated)	90%

Preparation of CPS: *At least* 30 minutes prior to processing, use a sterile, disposable 15ml or 50ml container to prepare CPS. Mixing of DMSO and FBS is an exothermic reaction. CPS must be prepared advance and chilled in the refrigerator (2°-8°C) or in an ice bath for at least 30 minutes prior to use. Discard any unused CPS after 8 hours.

Use the formula below to estimate the volume of CPS needed.

Examples:

Usable Whole Blood x	Average Cell Yield x	Freeze-down Concentration =	Estimated CPS
(10ml) x	(1.5x106 cells/1ml) x	(1ml/15x106 cells) =	1ml
(85ml) x	(1.5x106 cells/1ml) x	(1ml/15x106 cells) =	~9ml

Record CPS, DMSO and FBS volumes on the PBMC processing worksheet.

4. Counting Reagents

The requirements for counting reagents will vary depending on the method used. See the instructions for the method you will be using for the necessary cell counting reagent.

Calibration

1 No calibration is required for the processing steps.
2 Follow the applicable laboratory calibration procedures if using an automated cell counter.

Quality Control

1. Cell Yields

Cell yields are fairly consistent within populations. Infant populations typically generate higher lymphocyte yields than adult populations. Similarly, patients with AIDS or advanced HIV disease may be lymphopenic. It is important to be aware of the expected recovery that should be obtained for the population of participants for which the processing is being done. Based on this consistency, the cell yields can serve as internal quality control markers for each run. Yields outside the expected ranges may indicate a procedural error, reagent deterioration, cell count error, or calculation error.
The recommendations provided below are meant to provide a guideline to help

identify egregious technical errors prior to cryopreservation.

1.1 Expected Cell Yields

In most adult populations, the expected cell yield is approximately $1.5x10^6$ cells/mL with a range of 1-3 $x10^6$ cells/mL of usable whole blood volume.

1.2 Unexpected Cell Yields

- If cell yields are below $0.8x10^6$ or above $3x10^6$ cells/ml of usable blood, make a fresh dilution and re-count to rule out dilution or counting error.
- If the cell recount is consistent with the original cell count, record both cell counts on the PBMC Processing Worksheet and continue processing.
- If the recount is inconsistent with the original cell count, make another fresh dilution and recount a third time. If two of the three counts fall within your laboratory's acceptable precision limits, use the average of the two closest results for your calculations. If the imprecision persists, consult your supervisor.
- Record all results and any problems that occur during processing on the PBMC Processing Worksheet.

2. Cell Viability

Fresh PBMC cell viability is fairly consistent. Long processing time, poor technique and occasionally a specific participant specimen may adversely affect the viability.

2.1 Expected Fresh PBMC Viability

Freshly isolated PBMC viability should be >95%. If the cell counting technique allows the capture of viable cells, calculate and record the %viable cells on the worksheet.

2.2 Unexpected Fresh PBMC Viability

If the fresh PBMC viability is <95%, review the results with your supervisor and document on the PBMC worksheet.

3. Handling Times

Handling times can adversely affect cell recovery and viability. The collection, handling and processing times are collected on the PBMC worksheet.

3.1 Expected Times

The total handling time from collection to initiation of controlled-rate freezing should be less than 8 hours.
The actual processing time from introduction of fresh blood into the density gradient tubes until initiation of controlled-rate freezing should be completed within 2-3 hours to reduce the stress and exposure of the cells.

3.2 Unexpected (Long) Processing times

Review any long processing times with your supervisor and document on the

PBMC worksheet.

PBMC Processing

Note: Although the following describes procedures using the Accuspin tubes, other cell separation tubes with frit barriers may be used.

1. Separation of lymphocytes from peripheral blood using Accuspin separation tubes

1.1 All pipetting and mixing takes place in a biological safety cabinet, level 2 or greater.

1.2 Spray down all surfaces, racks, and reagent bottles with 70% ethanol prior to entering and using the BSC Hood.

1.3 Unless otherwise noted, the procedure is carried out at room temperature (1530°C).

1.4 Use a new pipet for each participant identification number (PTID).

2. Prepare whole blood samples, reagents, and supplies.

2.1 Prior to processing, prepare and chill the CPS.

2.2 Allow the Accuspin tubes to come to room temperature (15-30°C). See the Reagent section of this document for more information.

2.3 Record on the PBMC Processing Worksheet: the PTID, visit number, protocol, collection date/time, processing start date/time, lot numbers and expiration dates of all reagents, CPS, DMSO, and FBS volumes.

2.4 Before adding the blood, visually check the Accuspin tubes to see if there is liquid above the frit. If there is liquid above the frit, centrifuge the Accuspin tubes at 1000 x g for 30 seconds.

2.5 Carefully check the PTID on all tubes of blood received. Place all tubes for each PTID in one rack. Do not mix two PTIDs or tube types in the same rack.

2.6 If the specimen tubes are cold to the touch (due to cold ambient conditions such as transport in cooler months), allow the tubes to reach room temperature (15-30°C) before processing.

- **Plasma Replacement**

Perform this plasma replacement step ***only*** if plasma aliquots are required per protocol instructions. If plasma aliquots are not required, skip this step and proceed to step 4.

3.1 Centrifuge the whole blood at 400 x g for 10 minutes

3.2 Mark the volume of plasma in each tube

3.3 Transfer plasma to a 15 or 50 mL centrifuge tube for clarification of cellular debris

3.4 Add sufficient quantity of HBSS to bring blood back to its original whole blood volume, mix gently and continue PBMC processing at step 4.

3.5 Complete the plasma processing by centrifuging the collected plasma at 1200 x g for 20 minutes with the brake on. This may be done at a later time when the centrifuge is not is use for PBMC processing.

3.6 Aliquot spun plasma into labeled aliquot tubes as specified by protocol and discard any cellular debris in spun plasma tube.

4. Blood Dilution for Accuspin separation

Note 1: An accurate measurement of the usable blood volume must be determined and recorded on the PBMC worksheet. This can be accomplished by using a sterile pipette to transfer the whole blood to the Accuspin tubes and keeping track of the blood volume as it is pipetted, or by pooling the blood in a sterile graduated container before transferring to the Accuspin tubes and taking the measurement from the container.

Note 2: The final blood to HBSS ratio should be approximately 20ml blood:10ml HBSS or 2:1. The final combined volume above the frit in each Accuspin tube should not exceed 30ml. Use as many Accuspin tubes as required to distribute all of the blood for each PTID.

Note 3: Ficoll is toxic to cells; work quickly and efficiently during the separation steps.

4.1 Label each Accuspin tube with the PTID or appropriate lab identifier. You will need 1 tube for each 20ml of whole blood.

4.2 Uncap the tubes of anti-coagulated blood.

4.3 If a tube is grossly clotted (see glossary), discard it.

4.4 Document the type of specimen received, the total number of tubes discarded and the blood condition on the PBMC Processing Worksheet.

4.5 Using a sterile pipette, add 5ml of HBSS to each accuspin tube.

4.6 Mix whole blood gently then using a sterile pipette, transfer 10-20ml of blood into the labeled Accuspin tubes.

4.7 Using a sterile pipette, rinse each original anti-coagulated blood tube with HBSS, add rinse volumes to balance the Accuspin tubes making sure not to exceed the 30ml total tube volume (HBSS + Whole Blood).

4.8 Record the volume of usable blood on the **PBMC Processing Worksheet**. *Note:* Do not estimate the volume of usable blood based on the tube size. *Note:* Do not include the volume of HBSS used to dilute the blood sample.

4.9 Carefully cap the Accuspin tubes.

5. Accuspin density centrifugation and collection

5.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

5.2 Centrifuge at 800-1000 x g for 15 minutes at 18-26°C with the Brake OFF.
Note: PBMC separation may be improved for some specimens by centrifuging at

1000 x g.

Note: If the brake is on, it will disrupt the layers. Refer to the Calculations section of this document to convert g to rpm for your rotor length.

5.3 Prepare the same number of new sterile 50ml conical tubes as Accuspin tubes used in the separation centrifugation step.

5.4 Label each tube with the PTID. Use these new tubes for the next wash.

5.5 Gently remove the Accuspin tubes from the centrifuge so as not to disturb the layers. The centrifugation should result in six distinct layers in the tube from top to bottom:

5.6 Centrifugation results in the tube contents dividing into six distinct layers including the frit. From the top of the tube, these are:

- Plasma + HBSS
- PBMC layer
- Ficoll
- Frit
- Ficoll
- Packed red blood cells (RBC) and granulocytes

Inspect the tubes for hemolysis or small clots visible on the Accuspin frit that had not previously been noted and document them on the processing worksheet.

5.7 Re-evaluate the blood condition.

Note: Look for hemolysis, or clots after centrifugation. Grade hemolysis +1 through +4 based on the description given in the glossary. Record your observations in the comments section of the PBMC Processing Worksheet.

5.8 Using a new sterile pipette (serological or transfer pipette) for each PTID, remove the upper yellowish, plasma-HBSS fraction down to within approximately 2 cm (5ml) of the cloudy-white PBMC band located at the interface between the plasma-HBSS (yellowish) fraction and the clear separation medium solution. Discard the plasma-HBSS fraction per laboratory policy.

5.9 Using a sterile serological or transfer pipette, collect all cells at the cloudy-white interface above the frit. Use the tip of the pipette to gently scratch the inner walls of the tube at the interface to collect any cells stuck to the tube. Take care not to aspirate any more separation medium solution than necessary.

5.10 Transfer the collected cells from one Accuspin tube to a single corresponding, pre-labeled, sterile 50ml conical tube.

5.11 Re-cap the Accuspin tube containing the remaining red blood cells and separation media. Discard the Accuspin tube as biohazard waste following laboratory policy.

6. Wash 1:

6.1 Using a sterile 50ml pipette, add HBSS to each tube containing the collected cells to bring the final volume up to 45ml.

6.2 Re-cap all of the harvested cell tubes.

6.3 Centrifuge diluted cells at 250 x g for 10 minutes at 18-26°C with the brake on.

6.4 Remove the tubes from the centrifuge and check for the cell pellet.

6.5 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuging the tube. Document the problem and actions taken in the comments section of the PBMC Processing Worksheet.

If the cell pellet is still not visible after re-centrifuging the tube, document, remove and discard the HBSS supernatant and proceed.

7. Wash 2:

7.1 Re-suspend the pellet in a small volume (≤5ml) of HBSS mixing gently, but thoroughly, into a homogenous cell suspension.

7.2 Combine up to four pellet suspensions from the same donor into one 50ml conical tube. This is your harvested cell tube.

7.3 Use a small volume of HBSS to rinse the tubes from which the pellets were transferred.

Note: The combined rinse and harvested volume should not exceed 45ml.

7.4 Collect the HBSS rinse in the harvested cell tube.

7.5 Re-cap the tubes and place the tubes in the centrifuge.

7.6 Centrifuge at 250 x g for 10 minutes at 18-26°C with the brake on.

7.7 Remove the tubes from the centrifuge and check for the cell pellet.

7.8 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tube. Document the problem and actions taken.

If the cell pellet is still not visible after re-centrifuging the tube, document, remove and discard the HBSS supernatant and proceed.

8. PBMC Cell Count

8.1 Record the Counting method used on the PBMC Processing Worksheet,

8.2 Calculate and record on the processing worksheet, the HBSS counting re-suspension volume (V). This is the volume on which the cell count is based.

Note: The re-suspension volume should be approximately 20% of the usable whole blood volume rounded to the nearest ml.

8.3 If there is more than one pellet, use ½ of the counting re-suspension volume of HBSS to gently re-suspend and combine the cell pellets into one tube. Using the remaining volume, rinse the tubes from which the cells were transferred. Add the rinse to the harvested cell tube.

8.4 Complete the cell count using the SOP for the cell counting method approved at your laboratory.

8.5 Mix cells gently, but thoroughly, before sampling for your count.

8.6 Transfer a small volume of the re-suspension to a small tube for counting.

Note: If repeated counts are necessary, minimize the sampling volume needed.

8.7 Follow the SOP for the cell counting method approved at your laboratory and the PBMC Processing Worksheet to determine the cell concentration x 10^6 per ml.

Note: Cells at 10^3/µL = cells at 10^6/ml.

8.8 Using the appropriate Cell Counts section for your method on the back of the PBMC Processing Worksheet, record the cell count concentrations for each PTID (cells x 10^6 per ml).

Note: Automated counts may be run once. Manual counts should count at least the four large corner squares (1mm^2).

8.9 On the front of the PBMC Processing Worksheet, record the automated or manual cell count.

8.10 Calculate the total number of cells using the following formula:

$T = C \times V$ T = Total number of cells C = Concentration (10^6/ml) determined in counting method V = Count re-suspension volume of HBSS in ml

8.11 Record the total number of cells (T) on the PBMC Processing Worksheet.

8.12 Calculate the cell yield in cells/ml of usable whole blood using the formula below.

Cell Yield (10^6 cells/ml) = T /Usable Whole Blood Volume

8.13 Record the cell yield on the PBMC Processing Worksheet.

Note: The cell yield is calculated for quality purposes only. Refer to the Quality Control section of this document for the expected range of cell yields. If the cell yield is outside of the expected range, re-dilute and re-count following Quality Control guidelines.

8.14 Record any anomalies in processing in the comments section on the PBMC Processing Worksheet.

9. Quality Control

9.1 If the cell yield is outside the range of 0.8 to 3.0 x10^6 cells/mL, make a fresh dilution and re-count to rule out dilution or counting error.

9.2 If the cell recount is consistent with the original cell count, record both cell counts on the PPBMC Processing Worksheet and continue processing.

9.3 If the recount is inconsistent with the original cell count, make another fresh dilution and recount a third time. If two of the three counts fall within our laboratory's acceptable precision limits, use the average of the two closest results for your calculations. If the imprecision persists, consult your supervisor.

9.4 Record all results and any problems that occur during processing on the PBMC Processing Worksheet.

10. Final re-suspension volume and concentration for freeze-down

10.1 Calculate the CPS freeze-down re-suspension volume required for a desired final cell concentration range between 15-18 x 10^6 cells/ml by completing the steps below.

Note: The accepted range of cells/vial must fall between 10-20 x10^6 cells.

Calculate the estimated CPS freeze-down re-suspension volume (V1) required by using a desired final cell concentration of 15 x 10^6 cells/ml at one ml per vial.

V1 = (T/N1) x V2

T = Total number of cells

N1 = Desired number of cells = 15 x 10^6 cells per vial

V2 = 1ml per vial

Record the estimated volume (V1) on the PBMC Processing Worksheet.

Round V1 down to the nearest whole ml to determine the actual CPS re-suspension Volume (Vf). The acceptable range of cells/vial is 10-20 x 10^6 cells.

Note: PBMC aliquot volume = 1ml/vial. The milliliters of CPS will

equal the number of vials required.

Record the Actual CPS re-suspension volume (Vf) on the PBMC Processing Worksheet.

10.2 Calculate the actual number of cells per vial (N2) using the actual CPS freeze-down volume (Vf) determined in the previous calculation.

$N2 = (T / Vf) \times V2$

N2 = Actual number of cell per vial

T = Total number of cells

V2= 1 ml per vial

Record the final number of cells per vial (N2) on the PBMC Processing Worksheet.

Confirm that the PBMC Processing Worksheet is complete and that the calculations are correct.

11. Labeling

11.1 Complete the printing and labeling of the cryovials PRIOR to the final centrifugation.
Note: This is important to ensure that cells do not sit in a pellet for an extended period of time.

11.2 Cryovial labels will be generated using the Laboratory Data Management System.

11.3 Apply the labels on the cryovials so that the information can be easily read and the contents of the tube can be clearly seen.

12. Final Centrifugation

12.1 Place the harvested cell tube in the centrifuge.
12.2 Centrifuge the cells at 250 x g for 10 minutes at 18-26°C with the brake on.
12.3 Verify that all cryovials are labeled and available in your workspace.

13. Aliquoting for cryopreservation

Note: The following steps should be performed quickly to preserve cell integrity. It is permissible (although not necessary) to work on wet ice.

13.1 Remove and discard the HBSS supernatant. Keep the pellet.

13.2 Re-suspend the pellet using the calculated volume of cold CPS (Vf).

13.3 ***Flick*** the cell pellet prior to adding the CPS to re-suspend the cells.

13.4 ***Gently*** add the CPS to the re-suspended cells.

13.5 Work **quickly** once the CPS has been added. Do not allow the cells to sit in the freezing solution for longer than 10 minutes.

13.6 Aliquot 1ml per tube, evenly distribute any excess volume (due to the cell pellet size) among all of the tubes for that PTID.

14. Overnight controlled-rate freezing

14.1 Select the freezing method available in your laboratory. Follow the manufacturer's guidelines for use of StrataCooler or Mr. Frosty. *Note:* Before transferring cryovials to the SrataCooler or Mr. Frosty, the containers must be cooled to 2-8°C. Do not place cryovials in containers cooled to temperatures below 2°C.

Follow the appropriate on-site SOP for Cryomed.

14.2 Immediately transfer all cryovials to the controlled-rate freezing container.

For Mr. Frosty and StrataCooler, close the container and place it in a –70/-80°C freezer.

For Cryomed, start the cooling program.

14.3 Record the date and time at which the cryovials were moved into the –70/-80°C freezer on the PBMC Processing Worksheet.

Note: This is the completion time of processing.

14.4 Record the actual number of cryovials frozen on the PBMC Processing Worksheet.

15. Transfer of PBMC cryovials at –70/-80°C – Short Term Freezer Storage

15.1 Transfer the cryovials from the controlled-rate cooling container to the designated storage location at -70/-80°C. Store between -65°C to -94°C until shipped. *Note: Do not store in liquid nitrogen.*

For StrataCooler or Mr. Frosty, transfer the cryovials after 24 hours at –70/-80°C.

Note: Allow StrataCooler to equilibrate to 2-8°C prior to next use and Mr Frosty to equilibrate to room temperature prior to next use.

If you are using Cryomed, transfer the cryovials upon completion of the program to the –70/-80°C freezer.

15.2 Record the initials of the person making the transfer and the date/time of the transfer on the PBMC Processing Worksheet.

15.3 Record the appropriate storage information on the PBMC Processing Worksheet based on your laboratory's defined storage program.

16. Final Worksheet Review

16.1 The technician should confirm that the PBMC Processing Worksheet is complete and that the calculations are correct.

16.2 A second reviewer checks the worksheet for completeness and accuracy and then initials and dates the PBMC Processing Worksheet.

Note: All reviews should occur within two days of processing.

16.3 Store the PBMC Processing Worksheet according to laboratory policy.

17. Short-Term Freezer Storage & Shipping Guidelines

17.1 Acceptable short-term temperature range for PBMC storage is -65°C to -94°C for up to three weeks (21 days) from the date of freezing.

17.2 Dry-ice shipments must be shipped to the central specimen repository within 16 days from the date of freezing to allow safe delivery to CSR within 21 days for liquid nitrogen storage.

17.3 Contact HTVN international laboratory operations personnel if samples cannot reach their final destination within 21 days from the date of freezing. Permission to move samples to liquid nitrogen storage and ship in liquid nitrogen shippers is needed if the 21 day deadline cannot be met.

18. Long-term liquid nitrogen storage

18.1 Do not move PBMCs into liquid nitrogen storage without consulting HTVN International Laboratory Operations Division.

18.2 Frozen PBMC samples can be stored safely in liquid nitrogen indefinitely (vapor phase preferred).

18.3 Once samples have been stored in liquid nitrogen, all transfers or shipments must be maintained in liquid nitrogen (≤ –140°C).

18.4 Once samples have been stored in liquid nitrogen, samples cannot be shipped on dry ice.

18.5 Do not temporarily store samples in liquid nitrogen.

18.6 Never transfer samples from liquid nitrogen back to -70°C or -80°C freezers.

18.7 Once samples are stored in liquid nitrogen, the samples must remain in liquid nitrogen until thawed for use, including during shipping.

This marks the end of processing and storage. Follow the appropriate laboratory procedures for preparation and processing of shipments.

Reporting Results

Frozen PBMC is reported as PBMC cells per 1ml in CPS.

1 Original results are reported on the PBMC Processing Worksheet.
2 Upon completion of processing, the processor verifies that the PBMC Processing Worksheet is complete and that the calculations are correct.
3 A second review should catch and correct processing errors before specimens leave the site. This review should take place within 2 days of the PBMC processing.
4 After the second review, the PBMC Processing Worksheet is faxed to the International Laboratory Program QA Associate at 206-667-1069 or 206-667-6366.
5 Data is entered into the Laboratory Data Management System for the generation of cryovial labels and shipping manifest requirements.
6 Deviations and amendments are reported according to laboratory protocol.

Calculations

1 The RPMs are usually read off a nomogram chart. Nomogram charts are often included in the centrifuge maintenance manual. Be sure to use centrifuge and rotor specific charts.
2 It is recommended that you post the g to RPM conversion on your centrifuge for easy reference.
3 If you are unable to find a nomogram chart, g's can be converted to RPM's using the following formula.

$$RPM^2 = g/(1.18 \times 10^{-5})(r)$$

r = radius of rotor

g = relative centrifugal force expressed in units of gravity

RPM = revolutions per minute

Limitations of the Procedure

1 Optimum handling time of fresh blood for PBMCs is <8 hours from the time of collection. Cell function may drop for older specimens.
2 Optimum processing time for PBMCs is <3 hours from the time of adding blood to the cell separation tubes (Accuspin or equivalent) to the initiation of the controlled rate freezing cycle.
3 Studies indicate that specimens collected in an EDTA anti-coagulant give lower yields over time.
4 Avoid removing excess amounts of the separation media with the PBMC band as that can increase granulocyte contamination.
5 Avoid removing excess supernatant with the PBMC band to limit contamination from plasma proteins.

Procedural Notes

1 If the plasma is very cloudy, it may be difficult to see the interface of the Ficoll gradient. It is possible to improve the collection of lymphocytes by removing most of the plasma above the interface with a 10ml pipet, leaving only 3-4ml remaining. This allows for better positioning of the tip of the pipet for collection of cells.
2 Liquid nitrogen vapor-phase storage is the space in the storage tank that is above the LN2 liquid at the bottom of the tank.
3 Modification of the centrifuge speed to 250 x g is based on the information provided by the Sigma-Aldrich Accuspin System-Histopaque-1077 package insert, dated 2003/09.

Glossary of Terms References

Term	Definition
Centrifuge Temperature	18-26°C
Clotted, Grossly	More than ¼ of the whole blood mass is clotted and there is very little free whole blood remaining.
Clot, Small	Small clots will not usually be seen in the whole blood tube, but can be seen on the separation tube frit after centrifugation.
CPS	Cryopreservation Solution
CSR	Central Specimen Repository
CSTFB	Cell Separation Tube with Frit Barrier
DG Media	Density Gradient Media
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
Hemolysis	A pink to red coloration of serum or plasma due to the lysis of red blood cells. Hemoysis is graded and reported according to the following scale: 1+ Pale pinkish-red color in serum or plasma 2+ Pinkish-red color in serum or plasma 3+ Dark pinkish-red color in serum or plasma

Term	Definition
	4+ Dark red mahogany color in serum or plasma
HI-FBS	Heat Inactivated Fetal Bovine Serum

Icteric	A green or orange tinted plasma suggesting the presence of increased bilirubin.
LDMS	Laboratory Data Management System
PBMC	Peripheral Blood Mononuclear Cells
PTID	Participant Identification Number
Room Temperature (RT)	15-30°C

1 CHAVI SOP for PBMC Isolation and Cryopreservation, CHAVI-A0001, 31Jan06.
2 Cross-Network PBMC Isolation and Cryopreservation, 4Nov2005
3 Sigma-Aldrich Accuspin System-Histopaque-1077, procedure number A6929/A7054/A0561, Dated 2003-09.
4 Cox J.H., DeSouza M., Ratto-Kim S., Ferrari G., Weinhold K.J., and Birx D.L. Cellular Immune assays for evaluation of Vaccine efficacy in developing countries. Manual of Clinical laboratory Immunology. Rose N.R., Hamilton R.G., Detrick B. Eds. (6th ed.) p.301-315 (2002).
5 Weinberg A., Betensky R., Zhang L., and Ray G. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus- infected patients. Clin. Diagn. Lab. Immunol. 5:804-807 (1998).
6 Islam D., Lindbert A., and Christensen B. Peripheral blood cells preparation influences the level of expression of leukocyte cell surface markers as assessed with quantitative multicolor flow cytometry. Cytometry 22:128-134 (1995).
7 Boyum A. Isolation of mononuclear cells and granulocytes from human blood, Scand. J. Clin. Lab. Invest. 21:77-89 (1968).

Section 16: Sexually Transmitted Infection Testing; Urine and Mucosal Specimen Collection and Testing

Sexually transmitted infections (STI) are typically detected using blood, urine, or swabs of mucosal surfaces. Urine specimens can also be used for urinalysis, pregnancy testing (hCG), and drug abuse testing. Cervical specimens are used for Pap smears and immune factor testing; vaginal specimens are used for wet mounts, bacterial/viral culture, and pH; rectal specimens are used for bacterial/viral culture, wet mounts, and immune factor testing.

Syphilis is screened using serum by the Rapid Plasma Reagin (RPR) assay. This is a non specific test which is then confirmed using a test specific for Treponema such as the (define) TPHA. Herpes Simplex Virus 2 (HSV-2) is frequently tested using serum by ELISA methods. In some studies, patients who have ulcerations on their genitals will have a swab taken which is stored and then sent for multiplex PCR for *Treponema pallidum,* HSV 1 & 2, and *Hemophilus ducreyi*.

Chlamydia trachomatis and *Neisseria gonorrhea* (CT/NG) testing can be performed using urine or swabs. The two main methods used by HPTN/MTN sites both involve DNA amplification: PCR and strand displacement assay (SDA).

Wet Mount assay is used for the diagnosis of bacterial vaginosis (the presence of clue cells), yeast, and *Trichomonas vaginalis* (which can also be detected by culture using the in-pouch method). Wet mounts must be processed within 30 minutes of collection because *T. vaginalis* loses motility quickly. Vaginal pH is another diagnostic criterion of BV; for most HPTN/MTN studies the pH paper required is supplied directly by the Network Laboratory. Some studies may also collect genital secretions (semen and vaginal fluid) for viral load testing.

Procedure: NIMH Laboratory Protocol for Chlamydia and Gonorrhea PCR (Roche)

Prepared by	Date Adopted	Supersedes Procedure #
Charlotte A. Gaydos, Dr.P.H.		

Review Date	Revision Date	Signature

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Principle:
The AMPLICOR CT/NG Test is based on 4 processes: 1) specimen preparation, 2) PCR amplification of target DNA which employs primers that are biotinylated, 3) hybridization of the amplicons to oligonucleotide probes which are specific for the amplified PCR products, and 4) detection of the probe-bound amplified PCR product by a colorimetric assay.

The assay is a multiplex test that allows amplification of *C. trachomatis* (CT) DNA, *N. gonorrhoeae* (NG) DNA, and Internal control (IC) DNA. The DNA sequences detected are: CT: 207 base pairs of the cryptic plasmid and NG: 201 base pairs of the putative cytosine DNA methyltransferase gene. The Master Mix in the kit to which prepared specimens are added contains the primers for the CT, NG, and IC, nucleotides, polymerase enzyme and buffers. For more detailed explanation of the exact, detailed principles, consult the package insert.

This assay should be performed in two separate areas (separate rooms desirable) of the laboratory as directed in the instructions: 1) Pre-Amplification (A. Specimen Preparation Area and B. Reagent Preparation Area required) and 2) Post-Amplification.

Specimen:
In general, swabs, which may be cervical, vaginal, or urethral, or first catch-urine are appropriate specimens. The specimens are treated with a detergent solution to extract the DNA from the specimens and a second detergent is then added to finish the specimen preparation.

Patient Preparation: FEMALE patients are instructed to collect a self-administered vaginal swab and place it in a pre-labeled empty capped 15 ml centrifuge tube (Corning). Collection kits with instructions and a diagram will be given to each patient.

MALE patients will be instructed to collect a first-catch (first 10-50 ml of the urine stream) urine specimen in a sterile urine cup and to pour 10-15 ml into a empty capped 15 ml centrifuge tube (Corning).

Specimen Type and Shipping:

FEMALE-Self-Administered Vaginal Swabs (SAS). The tubes with the SAS can be shipped with cold packs or at room temperature. (Called DRY-shipped SAS, because there is no liquid transport media). Swabs should be left dry during shipping until they arrive to the processing laboratory, preferably within 4 days. After "initial processing" the SAS maybe stored frozen at -20C or lower until "routine processing" and testing

MALE Urines. The urine specimen is stable for 24 hr. at room temperature. After 24 hr., they should be stored at 2-8C until processing, but must be processed within 8 days or stored at -20C or lower for up to 2 months. If shipped by overnight delivery at room temperature, they should be stored at 2-8C until shipping to insure the room temperature storage does not exceed 24 hr.

Handling Conditions: All specimens should be handled as if they are capable of transmitting infectious agents (i.e. gloves). Specimens must be shipped in compliance with all applicable local state, country regulations for the transport of etiologic agents.

Specimen Processing:

Perform in Pre-Amplification Area: Specimen Preparation Area .

FEMALE SAS:

A. Initial processing:

1. Upon arrival to the laboratory, add 1 ml of molecular grade water to each tube with a separate pipet tip and vortex well for 30 sec.
2. Remove the 1 ml of water containing the specimen material which came from the swab to a labeled 1.5 ml polypropylene tube for storage. (Do not use snap cap tubes). Discard the tube with the swab. The specimen may be frozen at -20C or lower at this point or specimen processing may continue to routine processing.

B. Routine Processing:

1. To an appropriate number of sterile 1.5 ml polypropylene tubes (no snap cap tubes) add 100 ul of CT/NG Lysis Buffer from the kit using a sterile pipet tip. Label tubes with patient identifying number.

2. Gently mix specimens by vortexing. For previously frozen specimens, thaw at room temperature. Take care to avoid contamination when removing caps. Add 100 ul of each specimen as processed above to each tube, vortex for 30 sec., and incubate at room temperature for 10 min.

3. Using a new aerosol-barrier pipet tip for each specimen, add 200 ul of CT/NG Specimen Diluent from the kit to each specimen. Recap tube and mix by vortexing.

4. Incubate at room temperature for 10 min.

5. Processed specimens may be kept at room temperature for up to 2 hours before test procedure. Otherwise store processed specimens at 2-8C for up to 7 days. (If stored at 2-8C, warm to room temperature and vortex before proceeding).

MALE URINE:

Routine Processing:

1. To an appropriate number of sterile labeled 1.5-2.0 ml polypropylene tubes (no snap cap tubes) add 500 ul of CT/NG Urine Wash Buffer from the kit.

2. Vortex the urine thoroughly 3-10 sec. (If urine was previously frozen, thaw at room temperature before vortexing). Continue processing even if a precipitate is present. Carefully remove caps from tubes, one at a time, taking care to avoid contaminating gloves.

3. Using a new aerosol-barrier pipet tip for each urine specimen, add 500 ul well mixed urine specimen to the appropriate labeled tube containing the Wash Buffer. Recap tubes and mix well by vortexing.

4. Incubate at 37C for 15 min.

5. Centrifuge at $\geq$ 12,500 x g for 5 min.

6. Pour off supernatant and blot each tube on a separate sheet of absorbent paper.

7. Using a new aerosol-barrier pipet tip for each specimen, add 250 ul of CT/NG Lysis Buffer from the kit. Recap and mix by vortexing.

8. Incubate tubes for 15 min at room temperature.

9. Using a new aerosol-barrier pipet tip for each specimen, add 250 ul of CT/NG Specimen Diluent from the kit to each tube. Recap tubes and mix well by vortexing.

10. Centrifuge tubes for 10 min at $\geq$ 12,500 x g.

11. Processed specimens may be kept at room temperature for up to 2 hr. before continuing with the assay. Otherwise, store processed specimens at 2-8C for up to 7 days.

12. If processed specimens were stored at 2-8C, they must be warmed to room temperature and vortexed before amplification. After vortexing, centrifuge the processed specimens for 10 min at $\geq$ 12,500 x g.

Equipment and Materials:

Equipment:

1. Vortex Mixer
2. Microcentrifuge (max RCF 16,000 x g)
3. Pre-amp 37C incubator or heating block.
4. Perkin Elmer GeneAmp PCR system 9600 thermal cycler.

5. Post-amp 37C incubator
6. Microwell plate washer.
7. Microwell plate reader (range = 0 to $\geq$ 3.00 A_{450}) and printer.
8. Tube Racks (Sarstedt 93.1428).

Materials:

Provided:

1. Amplicor CT/NG Specimen Preparation Kit (Urine Wash Buffer, Lysis Buffer, Specimen Diluent).
2. Amplicor CT/NG Amplification Kit (Master Mix, Internal control, CT+ control, NG + control).
3. Amplicor Detection Kit (Denaturation solution, CT/NG hybridization buffer, Avidin-HRP conjugate, Substrate A, Substrate B, Stop Reagent, CT Probe coated microwell plate, NG Probe coated microwell plate, Internal Control (IC) Probe coated microplate).

Required, but not Provided:

1. Consumables: MicroAmp Reaction Tubes, Caps, Base, Tray, and Retainer.
2. Plastic resealable bag.
3. Eppendorf Repeater pipet with 1.25 ml individually wrapped Combitip Reservoir tips.
4. Pipetors and micropipet tips with plugged tips (aerosol barrier); 50 ul, 100 ul, 200 ul, 250 ul, 500 ul, and 1000 ul. Duplicate sets are required, dedicated to 1) the pre-amp area and 2) the post-amp area.
5. Extended length (long) pipet aerosol barrier tips (50-100 ul).
6. Sterile transfer pipets, graduated.
7. Powerless latex or vinyl gloves.
8. Unplugged tips (100 ul) for post-area only.
9. 96-well strip ejector, Costar #2578.
10. Molecular grade water.
11. 5 ml Serological pipets.
12. Graduated cylinder (1 L).
13. 15 ml screw top conical centrifuge tubes (Corning), dacron, plastic shaft swabs (Fisher or Baxter), and urine collection cups.

Procedure:

Before starting procedure, turn on thermocycler to allow it to warm up. All reagents must be at ambient temperature before use. **USE EXTREME CARE WHEN PIPETING AND PREPARING REAGENTS TO ENSURE CROSS-CONTAMINATION DOES NOT TAKE PLACE. Separate pipetors, glove boxes, and separate lab coats should remain in either Pre-Amp or Post-Amp areas.** (i.e. Do not walk into the pre-amp area wearing a post-amp lab coat and gloves).

A. Reagent Preparation **Performed in Pre-Amplification Area: Reagent Preparation Area.**

1. Determine the appropriate number of reaction tubes needed for patient specimen and control tests. (It is recommended that 2 CT and 2 NG processed controls be run with each batch of specimens). Place tubes in specimen tray and lock with retainer.

2. Vortex the CT/NG IC tube. (This must be used even if detection of IC will not be done). Prepare working Master MIX by adding 100 ul of CT/NG IC to one vial of CT/NG Master Mix (enough for 32 amplifications). Recap the Working Master Mix vial and mix well by inverting 10-15 times or brief vortexing. Record the date of preparation on the vial (reagent is good for 4 weeks or until expiration date of reagent). Discard the IC vial.

3. Pipet 50 ul of Working Master Mix into each reaction tube using a repeater pipet and 1.25 ml combi-tip Reservoir or a micropipet with a plugged tip. (Do not recap the tubes now).

4. Place the tray containing the working Master Mix in a resealable plastic bag and seal. Place the required number of reaction tube caps (strips) in another plastic bag and seal. Move them to and store them until use in the to the **Pre-Amplification Area: Specimen Preparation Area**. The Working master Mix is stable for 48 hr. at 2-8C in reaction tubes sealed in the plastic bag.

B. Specimen Preparation and Testing (See above for specimen processing). **Performed in Pre-Amplification Area: Specimen Preparation Area**

Take the specimens that were previously processed and proceed with addition of samples to the amplification tubes. Remember, if processed specimens were stored at 2-8C, they must be warmed to room temperature and vortexed before amplification. After vortexing, centrifuge the processed specimens for 10 min at $\geq$ 12,500 x g before proceeding to sediment any precipitate.

1. Using a micropipet with an aerosol barrier tip, transfer 50 ul of each processed specimen to the appropriate reaction tube containing the Working Master Mix. Use a new aerosol barrier tip for each specimen. Be careful not to disturb the pellet, which may not be clearly visible. Record the position of each Processed Control on the tray map. Cap the tubes.

2. Store the remainder of the processed specimens at 2-8C in the event retesting is required. Any retesting must be performed within 7 days of specimen processing.

C. Control Preparation **Performed in Pre-Amplification Area: Specimen Preparation Area**

Note: working control must be prepared fresh each day the test is performed and can be used to prepare multiple processed controls but must be discarded at the end of the day.
Note: The CT+ control serves as both the positive CT control and the negative NG control. The NG+ control serves as both the positive NG control and the negative CT control. Thus both controls must be prepared and used, even if specimens will be only tested for CT or only for NG.

1. Prepare the following Working CT and NG controls.

a. CT: add 100 ul of CT control to a 1.5-2 ml polypropylene tube containing 1.0 ml of CT/NG Specimen Diluent. Mix well by vortexing. Store at room temperature and discard at he end of the day.

b. NG: add 100 ul of NG control to a 1.5-2 ml polypropylene tube containing 1.0 ml of CT/NG Specimen Diluent. Mix well by vortexing. Store at room temperature and discard at he end of the day.

2. If testing **urine**, prepare the following Processed CT+ and NG+ Controls.

a. Prepare Processed CT+ Urine Control by adding 250 ul CT/NG Lysis buffer to 250 ul of Working CT+ control and mix well by vortexing. Incubate at room temperature for 10 min. Store at room temperature and discard at the end of the day.

b. Prepare Processed NG+ Urine Control by adding 250 ul CT/NG Lysis buffer to 250 ul of Working NG+ control and mix well by vortexing. Incubate at room temperature for 10 min. Store at room temperature and discard at the end of the day.

3. If testing **swabs** transported dry (or in chlamydia transport media [CTM]) , prepare the following Processed CT+ and NG+ Controls.

a. Prepare Processed CT+ Urine Control by adding 100 ul molecular grade water (if using swabs transported dry), (or CTM if using swabs transported in CTM) to 100 ul CT/NG Lysis buffer. Add 200 ul Working CT+ control and mix well by vortexing. Incubate at room temperature for 10 min. Store at room temperature and discard at the end of the day.

b. Prepare Processed NG+ Urine Control by adding 100 ul molecular grade water (if using swabs transported dry), (or CTM if using swabs transported in CTM) to 100 ul CT/NG Lysis buffer. Add 200 ul Working NG+ control and mix well by vortexing. Incubate at room temperature for 10 min. Store at room temperature and discard at the end of the day.

Note: If testing both urines and swabs on the same run, it is necessary to prepare both sets of controls (swab controls and urine controls). Then each set of controls may be tested once, thus, providing two CT+ and NG+ controls per batch.

4. Using a micropipet with an aerosol barrier tip, transfer 50 ul of each processed control to the appropriate reaction tube containing the Working Master Mix. Record the position of each Processed Control on the tray map. Cap the tubes.

5. Move the prepared samples (patient specimens and controls) in the trays to the Amplification/Detection Area. These PCR-ready samples may be stored at 2-8 C for up to 16 hr.

D. Amplification and Detection: **Performed in Amplification/Detection Area**

D1. Amplification:

1. Place the tray/retainer assembly into the thermocycler sample block, making sure the notch in the tray is at the left of the block and that the rim is seated properly. Close the cover and seal using the turn screw handle.

2. Program the thermocycle for the amplification. For more programming details, consult the GenAmp 9600 User's Manual.

HOLD Program:	2 min. 50C	
HOLD Program:	5 min. 95C	
Cycle Program (35 cycles)		10 sec. 91C; 50 sec, 62C; 35 sec, 72C
HOLD Program:	5 min. 72C	
HOLD Program:	72C forever	

In the thermocycler program, the ramp times should be left at the default setting (0.00), which is the maximum rate, and the allowed set point error at the default setting (2C). Link the 5 programs together into a METHOD program.

3. Start the METHOD program, which runs approximately 2 hr. Specimens may be removed at any time during the final HOLD program, but must be removed within 24 hr.

4. Remove the tray/retainer assembly containing the completed PCR amplification reaction from the thermocycler and place in the MicroAmp base. **Do not bring amplified DNA into the Pre-Amp area. This is a major potential source of contamination.** Remove the caps carefully to avoid aerosolization of the contents. Immediately proceed to the next step.

5. **Immediately** pipet 100 ul of **Denaturation Solution** to the first column of reaction tubes using a multichannel pipet with plugged tips and mix by pipetting up and down. **Discard tips and obtain a fresh set**. Proceed with this denaturation step row by row, using fresh tips each row. Incubate for 10 min. at room temperature to allow for complete denaturation of the DNA.

6. Store denatured amplicons at room temperature only if the detection step will be performed within 1-2 hr. Otherwise, recap the tubes using new caps and store plate at 2-8C for up to 1 week until the detection assay is performed.

D2. Detection

This procedure is to be followed for the detection of CT, NG, and IC, with each detection procedure using a different probe (CT probe, NG probe, and IC probe) coated microwell plate (MWP). All assays use the CT/NG hybridization buffer.

1. Warm all reagents to room temperature.

2. Prepare the Working Wash solution by adding 1 volume of 10X Wash concentrate to 9 volumes of distilled or deionized water and mix well. For manual washing prepare 40 ml of Working Wash Solution and for automated washing prepare enough according to the washer being used. The 1X Working Wash Solution can be stored at 2-25C in a clean plastic container and is stable for 2 weeks.

3. Allow the MWPs to warm to room temperature, before removing from foil pouch. Remove the appropriate number of 8-well strips from each foil packet (CT, NG, IC) for the number of specimens and controls being detected at one well of each type for each type detection (CT, NG, IC). Reseal the pouch containing the unused strips and the desiccant pillow. Set the strips into the plastic frame, candling carefully to avoid breakage. To remove strips from the frames, center the MWP on top of the Costar 96-well strip ejector and press down evenly on the corners of the frames. To lock strips in place, place the Costar ejector on top of the strips and press uniformly against the strips.

4. Add 100 ul of **CT/NG Hybridization Buffer** to each well to be tested on the MWP.

5. If the denatured amplicons were stored at 2-8C, incubate them at 37C for 2-4 min. in order to reduce viscosity.

6. Using plugged pipet tips and a multichanel pipet, pipet 25 ul of denatured amplicons into the appropriate wells of each of the MWPs containing the CT/NG Hybridization buffer. (Use same row/columns as PCR plate, going row by row and changing tips between rows). Gently tap the plate 10-15 times to insure sufficient mixing and a color change is seen from blue to light yellow.

7. Cover the MWP with the microwell lid and incubate the MWP for 1 hr. at 37C. ($\pm$ 2C).

8. Wash plate 5 times manually or by using a microwell plate washer using the Working Wash Solution (WWS). (Manual Wash: Empty contents of plate and tap dry on paper towels, Pipet 400-450 ul WWS to top of wells, soak for 30 sec., empty contents, tap dry. Repeat 4 times. (Automated Wash: Program washer to aspirate contents of wells, fill each well with WWS, approximately 350-450 ul, soak for 30 sec., aspirate dry. Repeat 4 times, tap the plate dry.

9.Add 100 ul **Avidin-HRP** conjugate to each well. Cover plate and incubate 15 min. at 37C ($\pm$ 2C).

10. Wash plate as in step 8.

11. Prepare Working Substrate by mixing 2.0 ml. of **Substrate A** and 0.5 ml of **Substrate B** for each multiple of two 8-well strips (16 tests). Prepare this reagent no more than 3 hr. before use and preferably immediately just before use. Store at room temperature and protect from exposure to direct light. [Full 96-well plate:12 ml of Substrate A and 3 ml of Substrate B].

12. Pipet 100 ul of prepared working substrate into each well being tested using a multichannel pipet and changing tips between rows.

13. Allow color to develop for 10 min exactly at room temperature in the dark. (Plate can be covered with a box).

14. Add 100 ul **Stop Reagent** to each well using the multichanel pipet and changing tips between each row.

15. Measure the optical density at A_{450} within 1 hr.

Quality control

Each PCR run must contain 2 Processed CT+ controls and 2 Processed NG+ controls. If testing only swabs transported dry (or in CTM) on the PCR run, prepare the appropriate set of controls (see Procedure C 3) and run in duplicate. If testing only urines, prepare the appropriate set of controls (see Procedure C 2) and run in duplicate. The CT + serves as the negative control for NG and the NG+ serves as the negative control for the CT. If testing both swabs and urines on the same PCR run, it is necessary to prepare one set of controls for each sample type; each set of controls should be tested only once.

Negative controls:

CT: Negative Controls for CT on CT MWP: The assay result for each NG+ Control (which is the neg CT control) should be less than 0.20 A_{450}. If not, the run must be invalidated and the entire test procedure from specimen preparation to detection must be repeated.

NG: Negative controls for NG on NG MWP: The assay result for each CT+ Control (which is the neg NG control) should be less than 0.20 A_{450}. If not, the run must be invalidated and the entire test procedure from specimen preparation to detection must be repeated.

If negative controls are consistently above 0.2, technical assistance may be needed and the Roche Technical Representative should be contacted.

Positive Controls:

CT: Positive controls for CT on the CT MWP: The assay result for each Processed CT+ Control on the CT MWP should be greater than or equal to 2.0 A_{450}. If not, the entire run must be invalidated and the entire test procedure from specimen preparation to detection must be repeated.

NG: Positive controls for NG on the NG MWP: The assay result for each Processed NG+ Control on the NG MWP should be greater than or equal to 1.5 A_{450}. If not, the entire run must be invalidated and the entire test procedure from specimen preparation to detection must be repeated.

If positive controls are consistently below the expected result, technical assistance may be needed and the Roche Technical Representative should be contacted.

Results:

CT:

Interpretation of Results.

1. Check control values to assure that the run is valid. (See Quality Control).
2. For a valid run, for a value greater than or equal to 0.8 A_{450} the specimen is reported as **positive** for CT, regardless of the value of the IC result.
3. CT value < 0.2 AND where the IC value is ≥ 0.2, specimen is reported as **negative** for CT .
4. CT value < 0.2 AND where the IC value is ≤ 0.2, **invalid negative** result (specimen showing inhibition of positive control). Process another aliquot of the specimen and rerun. (Processed sample may be diluted 1:10 and rerun both diluted and undiluted to try to get rid of the inhibition).
5. Specimens with values in the gray zone (≥ 0.2, <0.8), must have duplicate repeat testing performed on the processed specimen, regardless of the IC result. The final test interpretation of these specimens should be determined using 0.20 as the cutoff.

Repeat CT Result	Repeat IC Result	Interpretation
At least 1 result ≥ 0.2	Any value	Positive for CT
Both results <0.2	Both results ≥ 0.2	Negative for CT
Both results <0.2	At least 1 result < 0.2	Invalid negative, Indeterminate for CT

NG:

Interpretation of Results. [Note these interpretations differ slightly from the package insert due to the use of SAS].

1. Check control values to assure that the run is valid. (See Quality Control).
2. For a valid run, for a value greater than or equal to 2.5. A_{450} the specimen is **positive** for NG, regardless of the value of the IC result.
3. NG value < 0.2 AND where the IC value is ≥ 0.2, specimen is reported as **negative** for NG .
4. NG value < 0.2 AND where the IC value is ≤ 0.2, **invalid negative** result (specimen showing inhibition of positive control). Process another aliquot of the specimen and rerun. (Processed sample may be diluted 1:10 and rerun both diluted and undiluted to try to get rid of the inhibition).
5. Specimens with values in the gray zone (≥ 0.2, <2.5.), must have duplicate repeat testing performed on the processed specimen, regardless of the IC result. The final test interpretation of these specimens should be determined as **positive** if 2/3 results are > 2.0, regardless of the IC result.

Repeat NG Result	Repeat IC Result	Interpretation
At least 2/3 results $\geq$ 2.0	Any value	Positive for NG
Both results <2.0	Both results $\geq$ 0.2	Negative for NG
Both results <2.0	At least 1 result < 0.2	Invalid negative, Indeterminate for NG

Procedural Notes and Precautions:

1. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre+Amplification Area and moving to the Post-Amplification Area. Pre-Amp activities should begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each Pre-Amp activity and not used elsewhere. Post-Amp supplies must remain in the Post-Amp Area at all times.

2. As with any test procedure, good laboratory practice and proper training is essential to the proper performance of the test. Due to the extremely good analytical sensitivity of this test, extra caution and care should be taken to preserve the purity of kit reagents and to the performance of the assay.

Storage and Handling Requirements:

1. Do not freeze reagents.
2. CT/NG Master Mix and IC must be stored at 2-8C. Unopened, reagents are stable until expiration date indicated. Working master Mix is to be stored at 2-8C and is stable for 4 weeks.
3. Ct and NG controls must be stored at 2-8C and are stable until expiration date indicated.
4. CT/NG Lysis Buffer should be stored 2-25C. Ct/NG Specimen Diluent must be stored at 2-8C. If a precipitate forms in these reagents upon storage, warm to ambient temperature and mix thoroughly. These reagents are stable until expiration date on item.
5. The Ct/NG/IC Probe coated MWP must be stored at 2-8C in the foil pouches provided and are stable until expiration date provided. Once opened, the MWP is stable for 3 months (or until the expiration date, which ever comes first) in the sealed pouches.
6. Denaturation Solution, Hybridization Buffer, and Stop Reagent should be stored at 2-25C and are stable until expiration date noted.
7. Avudun-HRP Conjugate, Substrates A and B must be stored at 2-8C. Unopened they are stable until expiration date. Once opened, they are stable for 3 months (or expiration date, whichever comes first).
8. Working Substrate must be freshly prepared before use each time and protected from light (See Reagent Preparation) and as such is stable for 3 hr.. Do not expose Substrates A and B or Working Substrate to hetals, oxidization agents of direct light.
9. 10X Wash Concentrate should be stored at 2-25C. The solution is stable until the expiration date. Wash Solution (1X) should be stored at 2-25C and is stable 2 weeks from date of preparation.
10. Dispose of reagents and waste in accordance with country, federal, state and local regulations.

Procedural Limitations:

1. False negatives may occur due to polymerase inhibitors.
2. Assay will not detect the plasmid-free variants of CT, which are very rare.
3. Interference by endogenous and exogenous substances has not been established.
4. Detection of CT and NG is dependent on the number of organisms present in the specimen and may be affected by poor specimen collection methods.
5. Only the specimen types indicated should be tested. The assay is not validated for eye of respiratory specimens.
6. Therapeutic success or failure cannot be assessed by this assay.
7. Findings should be interpreted in view of all clinical and laboratory findings.
8. Use of this assay should be limited to those personnel thoroughly trained in the techniques of PCR.

Performance Characteristics:

Specificity: The analytical specificity has been evaluated against the most common genital organisms and viruses and showed no reactivity. See package insert for list.

Specificity: The analytical sensitivity of the CT test is one Inclusion Forming Unit (IFU) per PCR reaction for each of the 15 serovars of CT. The analytical sensitivity of the NG test is 5 colony forming units (CFU) per PCR reaction for all 15 isolates of NG tested.

See package insert for more performance characteristics and information regarding precision and clinical performance.

CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date: 15/March/2006 Author: J. Westerman, R. Reed
Title: GC/CT Strand Displacement Analysis (BDProbeTec ET)		Revised Effective Date: Author: Jennifer Westerman, MT(ASCP)
Proc#: V.2.9-3	File Name: V.2.9-3 GC-CT BD ProbeTec ET	Page 475 of 538

I. PURPOSE/PRINCIPLE: To provide a procedure for qualitative detection of Chlamydia trachomatis (CT) and Neisseria gonorrhoeae (GC) using the BDProbeTec ET. This test uses Strand Displacement Amplification (SDA) technology.

II. MATERIALS AND EQUIPMENT:

Bio-Safety Cabinet
BD ProbeTec ET Instrument
BD ProbeTec ET Pipettor
BD ProbeTec Pipette tips
BD ProbeTec ET Lysing Heater
BD ProbeTec ET Priming and Warming Heater
BD ProbeTec Lysing Rack
BD ProbeTec Instrument Plate
Centrifuge
Vortex
CT/GC Control Set
Refrigerator – Storage of Urine samples
Pipette (1-5mls) with tips
Powder Free Gloves
Sample tubes and caps
Sample Diluent
10% JIK
CT/GC/AC Reagent Pack
Accessories Kit (amplification sealers, priming covers, disposal bags)
Dacron/Polyester Swab

III. SPECIMEN TYPE / STORAGE

A. Urine

1. First stream Urine. See Urine collection SOP.
2. Minimum volume is 15 – 20 mls. Maximum volume is 60 mls.
3. Storage for 30 hours at room temp. (15 – 30 °C) and from up to 7 days at 2-8°C.
4. Urine can be stored at -20 °C for two months.
5. Urine received after 6 days will not be processed.
6. If the urine sample is bloody, compare the color of the urine to the laminated picture in the SOP manual. If the sample is equal to or more red than the picture the specimen is rejected and the clinic is notified.

B. Endocervical Swabs

1. Collect specimen using the BD Culturette Direct Swab. See Collection SOP.
2. Place swab into transport tube and cap tube.
3. Storage 4 – 6 days at 2-27 °C.
4. Swabs received after 6 days will not be processed.

C. Lysed Samples

1. Store at Room Temperature (18 – 30°C) for 6 hours.
2. Store for up to 5 days at 2 – 8 °C. Samples must be vortexed and re-lysed prior to testing.
3. Store up to 98 days at -20 °C. Samples must be thawed at room temperature, vortexed and re-lysed prior to testing. Lysed samples may be frozen and thawed twice.

D. Reagent and Kit Storage

1. CT/GC/AC Reagent pack is stored at 2-33 °C. Kit wells expire one month once the packet is opened.
2. Sample diluent is stored at 2-33 °C and used until the expiry date.

IV. FORMS:

V.2.9A ProbeTec Run Lay Out Form
V.2.9B Monthly Work Area Culture Log

V. PROCEDURE:

A. Preparing the system

1. Turn on the system components before beginning sample processing. This allows the system to warm up to standard operating temperatures while you are preparing samples. Be sure the following items are turned on:
   a. Lysing Heater
   b. Priming and warming Heater
   c. BDProbeTec ET instrument
2. The instrument requires 30 minutes to warm up, and the heaters 90 minutes.
3. Verify the pipettor program 3 and 5. The pipettor requires no warm up.
   a. Program 3:
      1) Fill 600ul
      2) Dispense 150 ul three times
      3) Purge
   b. Program 5:
      1) Fill 100 ul
      2) Dispense 100 ul
      3) Mix 50 ul
      4) Zero (flashes on and off)

B. Urine Sample Preparation and Processing

1. Samples are processed in the Bio-safety cabinet in the PCR Prep Room.
2. Mix the urine sample by swirling.
3. Label the sample tube with the Patient ID and LIMS access number.
4. Pipette 4.0 ml of urine sample into tube and place cap on the tube.
5. Process one urine sample at a time and recap prior to opening the next urine sample. **NOTE:** To avoid contamination do NOT have more than one urine open at the same time.
6. Insert the tube into the lysing rack.
7. Set timer for 10 minutes.
8. Insert the lysing rack into the lysing heater to pre-warm urine samples. Start timer.
9. Write the PTID and the LIMS access number on **V.2.9AProbeTec Run Lay Out Form**. This is the order they will be logged into the ProbeTec.
10. After timer beeps, remove the lysing rack from the heater and cool to room temperature (15-30 °C) for 15 minutes up to 6 hours.
11. Centrifuge at 2000 x g for 30 minutes using program 5 on the CL3 centrifuge in the specimen processing area.
12. During centrifugation **proceed to section D** to log in the samples into the ProbeTec.
13. Remove the sample tubes from the centrifuge and place in the lysing rack according to the plate layout report. Confirm specimen identification for each sample.
14. Place positive and negative controls in their assigned position.
15. Processing one urine sample tube at the time, decant the supernatant into the JIK disposal container.
16. Blot the last drop onto a piece of tissue.
17. Repeat step 15 and 16 for each sample.
18. Use a different area to blot each tube to keep from cross-contaminating samples.
19. Proceed to lyse the sample. See section E.

C. Quality Control

1. A positive and negative control is tested with each run and /or new kit lot number. For example, if the run contains more than one kit lot number a control set is tested with each lot number.
2. Control samples are placed into the last two wells. The Positive control is placed before the Negative control.
3. The CT/GC control must test correctly in order to report patient values.
4. If the controls fail the run fails and patient results will not be reported by the instrument.
5. The additional use of the amplification control is used to determine inhibition and is included for each patient sample and control. **(The amplification control is not required by FDA. Once a laboratory is familiar and proficient with this test the discontinuation of the amplification control should be considered.)**
6. Preparation of Controls:
   a. Remove the cap from the control sample.
   b. Using a new pipette tip, add 2 mls of diluent.
   c. Recap the tube and vortex for 5 seconds.
   d. Controls are ready to be lysed. Proceed to section F.
7. Testing Work Surfaces:
   a. Once per month, the work area and equipment surfaces are swabbed using a Dacron swab and tested for the presence of DNA contamination.
   b. Label a collection tube with area to be tested.
   c. Pipette 2mls of sample diluent into the tube.
   d. Take a clean swab and dip into sample diluent and wipe across the area to be tested.
   e. The swab is processed as an Endocervical swab. Proceed to section E. 5.
   f. Areas to be tested may include:
      1) Surface of the lysing heater
      2) Lysing rack
      3) Priming and warming heater
      4) Black microwell trays
      5) Pipettor handle
      6) Instrument keyboard
      7) Work bench
   g. Report results on **V.2.9B Monthly Work Area Culture Log**. Write date of testing in the box. If samples are negative tick the box. If positive see below. Don't tick the box until test results are negative.
   h. Positive results:
      1) Clean area with fresh 10% bleach followed by distilled water.
      2) Retest the area until a negative result is obtained.
      3) Document on II.4B Corrective Action Log positive cultures and actions taken.
8. **New Reagent Lot Number**:
   a. Run one patient positive and one patient negative sample when new lot number is started.
   b. Compare results to run using the previous lot number.

D. Logging in New Samples

1. From the Main status Screen, press the "plate operations" soft key. (Pictured are the pencil, eraser, and trashcan over a report).
2. To create a new plate, press "select a new plate" soft key. (Pictured is the pencil over a report).
3. At the Log-In screen the plate number is shown at the top left corner of the screen. The plate number defaults to the next unused plate number. (To add samples to an existing plate definition, see "Modifying an existing plate" in section K. You must know the plate number to modify, delete, or start testing on an existing plate.)
4. Press the "➔I" key to move from field to field.
5. In the kit (lot) number field, scan or type in the 7- or -9- digit kit barcode label number for the lot of reagents being used.
6. The system automatically enters the default test type field (CT/GC). Press the "➔I" key to accept the test type shown, or press the UP or DOWN ARROW to scroll through the available test types. When the desired test type appears, press the "➔I" key. Make sure the 3rd well has amplification control listed.
7. Using **V.2.9A ProbeTec Run Lay Out Form**, scan the barcode label or type in the specimen tube access number. When scanning the barcode label the system will automatically proceed to the next specimen ID. When typing in the PTID number you will need to press save (picture of diskette) to advance to the next specimen ID.
8. A positive and negative Quality control test MUST be run for each kit number (reagent lot) used on the plate. The controls are placed at the end of the plate. To log in a control, press the "QC" soft key.
   a. The cursor appears in the QC lot number field. Scan or type in the lot number for the controls.
   b. The control filed defaults to negative (QC-). Press the UP or DOWN arrow next to the screen to scroll through the choices. Press the 'save' soft key to save the control.
   c. Repeat step b. to log the other control.
9. To delete any of the logged in samples, press the 'move to next field" "➔I" soft key to advance the cursor to the plate location field.
   a. Use the UP or DOWN ARROW to increase or decrease the plate location until the desired location appears.
   b. Then press the "delete samples" soft key (Picture: erase over a report). Press the "confirm" (OK) soft key to save your deletion, or press the "cancel" (⃠OK)soft key to leave the plate unchanged.
10. When all samples on the plate have been logged in, print a Plate Layout Report by pressing the "print report" (picture of printer) soft key. Arrange samples in the lyse rack according to the plate layout report including the positive and negative controls.
    Note: Multiple plates may be logged in prior to testing, up to a total of 30 untested plates.
11. Return to the main screen by pressing the button with a door and arrow.

E. Lysing Preparation

1. Using the Plate Layout Report, place the samples into the lysing rack. Verify each sample and initial next to the sample ID on the Plate Layout Report.

2. Determine the required volume of sample diluent. (2mls per sample plus 4 mls for controls)
3. Pour the amount of sample diluent needed into a clean container. **Note**: Do **NOT** pipette from the diluent container. Do **NOT** pour diluent back into diluent container.
4. Urine Samples.
   a. Uncap one sample tube at the time; add 2 mls of sample diluent.
   b. Recap the sample tube and vortex sample for a minimum of 5 seconds.
   c. **Change pipette tip** and proceed to the next sample tube. **Note:** Do NOT have more than one sample tube uncapped during pipetting diluent.
   d. After sample diluent has been added to all samples **DISCARD remaining diluent**.
5. Endocervical Samples.
   a. Label sample tube with PTID number and cap sample diluent tube.
   b. Uncap one sample tube at the time; add 2 mls of sample diluent to the sample tube.
   c. Insert the swab and swirl in diluent 5-10 seconds
   d. Express swab and discard.
   e. Recap tube (using same cap) and vortex tube for 5 seconds. Proceed to next swab sample.
6. Have the supervisor or designee review the lysing rack with the plate layout report. The review will confirm the samples are in the designated position and the labeling is correct. The person reviewing will write their initials, date and time of review on the plate layout report.
7. Lock tubes in the lysing rack and place lysing rack in the lysing heater.
8. Set timer for 30 minutes.
9. Remove rack and allow samples to cool at room temperature for at least 15 minutes. See section III.C for storage temperatures and length of storage for lysed samples.

F. Prepare Priming Plate

1. Arrange the number of priming microwells (WHITE Pouch) in the instrument plate according to the plate layout.
2. The plate layout indicates the color of the priming well on the bottom of the layout report. **Note:** Fully close the packages containing the microwells to maintain expiration date.
3. Cover plate with plastic cover until ready for use.
4. Place paper towel in front of the lysing rack and dampen with 10% JIK (bleach) solution.
5. Remove the caps from the first row of lysed and cooled tubes. Discard the caps. **Do not cross cap over another sample to avoid contamination**!
6. **CHANGE GLOVES** to avoid contamination!
7. Uncover the priming wells of the samples to be pipetted.
8. Using the BDProbeTec ET Pipettor, pipette the samples to the priming wells using **Program 3**.
   a. Place the pipettor over the tips to be inserted.
   b. Press firmly to ensure the tips are on well.
   c. Raise pipettor up and take care not to touch tips.
   d. Pull the lever on the pipettor to expand the arrangement of the tips.

e. Insert tips into the open sample tubes and touch the bottom of the tubes.
f. Raise the tips slightly and press the trigger to aspirate the 600 mls of sample.
g. Wait for the beep. **Visually inspect** the volume of sample.
h. Move the pipettor over the waste bag and push the lever on the pipettor to shrink the tips.
i. Proceed to the Green (CT) priming wells for the samples.
j. Place the tips into the green priming wells at a slight angle.
k. Press the trigger to deliver 150 ul to the wells. Proceed to the Yellow (GC) wells and then the Black (AC) wells.
l. Discard tips.

9. Place **NEW caps** on the sample tubes.
10. Continue pipetting the remaining samples as in steps 5 through 8.
11. When the plate is complete, cover priming wells.
12. Start timer for 20 minutes. **Note: Samples are stable for 6 hours at room temperature. If samples are not processed within 6 hours the lysing step would need to be repeated.**

G. Prepare Amplification Plate

1. Arrange amplification microwells (SILVER Pouch) using the plate layout report.
2. After the 20 minute incubation, remove the cover on the priming plate and place on the priming heater.
3. IMMEDIATELY put the amplification microwell plate on the warming heater.
4. Start timer for 10 minutes.
5. Set pipettor to use program 5.
6. Set out seal for the plate.

H. Sample Transfer to Amplification Plate

1. Immediately after timer beeps, pipette samples from the priming microwells in column 1 to the amplification wells in column 1.
   a. Place pipette over tips and press firmly.
   b. Place pipette into the bottom of the sample microwells at a slight angle for pipetting.
   c. Press the trigger to aspirate 100 uls of sample.
   d. Move sample to the amplification microwells.
   e. Press the trigger to dispense the sample.
   f. Press the trigger to mix the sample. Look at tips to make sure all wells are mixing.
   g. Exit to the waste container.
   h. Press the trigger again to return to the beginning of the program.
   i. Discard tips and proceed to next column of amplification wells.
2. When all samples have been pipetted into the amplification microwells, **use a sealer to cover the plate**.
3. IMMEDIATELY place the plate into the BDProbeTec ET instrument and initiate the run. **Note: Timing is critical!**
4. Use a sealer to cover the Priming microwells.

5. Dispose of the Priming microwells in a zip lock disposal bag and discard in to the Biohazard bag.

I. Run

1. From the main status screen, press the "move carrier out" soft key.
2. When the "okay to open door" icon appears, open the door and lift the plate cover. Remove the BDProbeTec ET thermal test plate and place in the heater block. Place the plate on the carrier as soon as possible (it fits in one orientation only). **NOTE:** THE PLATE MUST BE SEALED BEFORE TESTING.
3. Lower the plate cover and then close the door.
4. Press the "start test" (flag with plate) soft key.
5. The plate list box appears .use the UP or DOWN ARROW to highlight the plate number you want to test. In the plate list box, the plate number, date, and time that the plate was defined is shown. When the desired plate is highlighted, press the "confirm" soft key to initiate testing. Make sure you select the number for the plate that has been placed on the carrier for testing.
6. The analysis will take 60 minutes.
7. The "testing in progress' icon appears while the plate is being tested. A timer counts down time remaining as testing progresses.
8. When the plate test is complete, the instrument beeps 3 times, the "test complete" icon appears, and the TEST COMPLETE indicator on the instrument illuminates.
9. Press the "carrier out" soft key. When the "okay to open door" icon appears, open the door and remove the plate. Press the "carrier in" soft key.
10. Disposal of Microwells:
    a. Dispose of the Amplification Microwells in a zip lock disposal bag. **Note: This step is very important to prevent contamination.**

J. Modifying and Deleting an Existing Plate

1. Modifying an existing plate enables you to add more samples if space is available or to delete samples. You cannot modify a plate after testing begins.
    a. To modify an existing plate, from the main status Screen, press the "plate operations" soft key.
    b. Press the 'modify plate" soft key.
    c. Use the UP or DOWN ARROW to highlight the plate number you want to modify. In the plate list box, the plate number, date, and time that the plate was defined is shown. When the desired plate is highlighted, press the "confirm" soft key to select that plate editing.
    d. The plate login screen appears. At this point, you can add, modify, or delete samples as described in section D. Log in New Samples.
2. To delete an existing plate, from the main status screen, press the "plate operations" you cannot delete a plate after testing begins.
    a. Press the "delete plate" soft key.
    b. Use the UP or DOWN ARROW to highlight the plate number you want to delete. In the plate list box, the plate number, date, and time that the plate was defined is shown. When the desired plate is highlighted, press the "confirm" soft key to DELETE that plate.

K. Aborting a Test - Note that if a test is aborted, you CANNOT re-initiate testing on that plate.

1. While the 'test in progress icon" with countdown timer is displayed, press the 'carrier out' soft key.
2. Press the 'confirm' soft key. This aborts the test in progress.
3. When the 'okay to open door' icon appears, open the door and remove the test plate

L. Responding to Alarms and Errors

1. As you perform activities at the BDProbeTec ET instrument, and as testing progresses, system alerts and errors may occur.
2. Different types of alerts and errors are flagged by one or more of the following: 'E' and 'W' error codes audible tones, the system Alert icon appearing on the LCD display, or the instrument's System Alert indicator illuminating. Generally, the more serious the condition, the more ways the system notifies you of the problem.

CAUTION: When the system notifies you of alerts and errors, you should respond to the condition immediately.

3. System alerts, which comprise all 'E' type error coded, are reported in the system alert list. These errors cause the system alert icon to appear on the Main Status Screen and the system Alert indicator to illuminate, and can be reviewed by pressing the 'system alert' soft key (see below). The errors must be reviewed to clear the system alert condition.
4. Activity errors comprise all 'W' type codes. These errors (such as attempting to exit the plate login screen without entering positive and negative quality control wells) cause the activity error icon to appear on the currently displayed screen. They do not put the system into alert condition; these errors can frequently be cleared by simply performing the activity correctly (such as entering the required controls before exiting the screen.
5. All the 'E' and 'W' error codes are discussed in detail in section 7.2- error messages of the Operations Manual. The audible tones are discussed in section 3/2/6- Audible Tones and Alarms. The audible alarm can be silenced by pressing the SILENCE ALARM key, if the alarm is a 'system alert' type of alarm (see section 3.2.6) - Audible Tones and Alarms).

VI. RESULTS/REPORTING:

A. Printing Reports

1. Assay Result Report- provides the test results of samples and controls on the plate whose testing has completed.
2. The Assay Result Report prints automatically at the end of a plate test, or by requesting from the main status screen.
   a. The 'print report' soft key appears only when you are in a screen or performing an operation in which there is a related report.

b. The Assay Result Report can only be printed from the Main Status screen.

3. The Assay Result Report can be reprinted by pressing the "print report" soft key from the main status screen and selecting the plate number to reprint. The results for the plate remain in the data base until all 30 available plate" slots" are defined and that plate number is reassigned by the system.

B. Resulting

1. **Negative Result** = MOTA count less than 2,000
2. **Low Positive Result** = MOTA count between 2,000 – 9,999
   a. Sample is repeated.
   b. If repeat result is <2000, the sample is resulted as indeterminate and a 2nd sample is requested.
3. **Positive Result** = MOTA count greater than or equal to 10,000
   a. Sample is repeated.
4. All plate layout reports are reviewed by a laboratory supervisor/designee prior to resulting patient/participant samples.
5. Test results are entered into the LIMS system using function **ERM** for both GC and CT.
   a. Type in the access number for the sample.
   b. Type **?** to see the result coded text for each test.
   c. Use the arrow key or click on the result of the sample.
   d. [ENTER] or click on OK.
   e. [F12] to save results.
7. CIDRZ Laboratory reports are automatically printed in batch when all results are complete.
8. The CIDRZ laboratory reports are then reviewed and compared to the ProbeTec printout prior to distribution to the clinic by the lab protocol supervisor/designee.

VII. LAB REFERENCE RANGE:

A. MOTA = Method other than acceleration. This refers to the summary of all the fluorescent counts.

B. Amplification Control = MOTA values should always be greater than 1,000. The amplification control is an indication of DNA present or the presence of inhibitors.

VIII. LIMITATIONS:

See package insert for limitations.

IX. SPECIAL NOTES:

- This test is done at the CIDRZ Central Laboratory
- This test is batched.
- TAT – 48 hours from testing.
- All low positive and positive samples are repeated. Areas or groups of positives are also re-assayed to verify result.

- All lysed samples are frozen at -80 °C after testing. Negative samples are stored for a minimum of one month. Positive samples are stored indefinitely.

X. REFERENCES:

BDProbeTec ET User's Training Manual. Becton, Dickinson and Company. November 1999.

Package insert. BD ProbeTec ET Chlamydia trachomatis and Neisseria gonorrhoeae Amplified DNA Assays. Sparks, MD: Becton, Dickinson and Company.

CIDRZ – Central Laboratory
Lusaka, Zambia

BD ProbeTec ET Monthly Maintenance Log

Year: _____ Equipment Serial Number: _____ Location: _____

Procedure	January	February	March	April	May	June	July	August	September	October	November	December
Monthly Maintenance												
1. Change or clean air filter at rear of instrument												
2.Check instrument temperature(52±0.8ºC)												
Periodic Maintenance (As Needed)												
1.Clean Pipettor												
2.Calibrate Pipettor												
3.Replace Pipettor Batteries												
Yearly Maintenance												
1.Normalizer replacement												
INITIALS												

Comments:_____
_____
_____
_____
_____ Reviewed by: _____ Date: _____

SOP: HERPES SIMPLEX VIRUS TYPE 1 AND TYPE 2 IgG, ELISA

PRINCIPLE

Herpes Simplex virus infections are caused by two distinct antigenic types, HSV-1 and HSV-2. Both HSV types are common human pathogens. HSV-1 is usually associated with infections in the oropharyngeal area and eyes while HSV-2 causes most genital and neonatal infections. However, the tissue specificity is not absolute. HSV-2 can be isolated occasionally from the oropharyngeal area and 5 to 10% of primary genital infections may be caused by HSV-1.

Serological procedures may be useful for diagnosis of primary HSV infections, and for determining evidence of past infection with HSV. Diagnosis of primary infection is based on demonstration of seroconversion or a significant rise in titer between paired acute and convalescent sera. Serological procedures are less useful for diagnosis of recurrent HSV infection since recurrent infections are often not reflected by a change in antibody levels. Also, among persons with a first time HSV-2 infection who experienced a previous childhood HSV-1 infection, little or no increase in HSV-2 type specific antibodies may be produced.

The MRL Diagnostics assay utilizes purified HSV recombinant gG1 antigens immobilized on polystyrene microwells.

The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in microwells with HSV antigen. Any antigen specific antibody in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase conjugated goat anti-human IgG is added to the wells and the plate is incubated. The conjugate will react with specific IgG antibody. The wells are washed to remove unreacted conjugate.
3. Microtiter wells containing immobilized peroxidase conjugate are incubated with enzyme substrate solution. Hydrolysis of the substrate by peroxidase produces a color change. After a period of time, the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution is directly related to the antibody concentration in the test sample.

SPECIMEN

Only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures should be used in this assay. No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic or bacterial contaminated sera. Sera should be stored at 2-8°C for no longer than 5 days. If delay in testing is anticipated, store test sera at -20°C or lower. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and give erroneous results.

REAGENTS--SPECIAL SUPPLIES AND EQUIPMENT

1. MRL Diagnostics HSV-1 (Catalog No. EL0910G) and HSV-2 IgG (Catalog No. EL 0920G). Store unopened kits at 2-8°C.

8.Antigen Wells, 96 wells

12 Eight-well polystyrene microwell strips on a frame. Each well is coated with recombinant gG1/gG2 antigen. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets. Unused strips should be immediately resealed with dessicant and returned to storage at 2-8°C.

9.HSV-1 IgG Conjugate, 12 mL

One vial of affinity-purified and peroxidase-conjugated goat anti-human IgG (Fc fragment specific). Contains 0.05% thimerosal as a preserative.

C. IgG High Positive Control, 0.14 mL

IgG Low Positive Control, 0.14 mL

One vial of human serum per each control level. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

Negative Control, 0.14 mL

F. IgG Cut-Off Calibrator, 0.25 mL

One vial of human serum per each control level. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

Sample diluent, 100 mL

One vial of protein, surfactant, and preservatives in PBS. Stable at 2 - 8°C until the expiration of the date stated on the label. Allow to warn to room temperature before use.

10X Wash Buffer, 100 mL

One vial of surfactant in PBS with 0.01% thimerosal. Prepare a 1X wash buffer solution before use.

PREPARATION OF REAGENT

To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.

Reagents--Special Supplies and Equipment (cont.)

Substrate Reagent, 12 mL

One vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. A dark blue color indicates contamination with peroxidase; and, if this occurs, use a fresh bottle. Store at 2-8°C.

Stop Reagent, 12 mL

One vial 1M sulfuric acid.

K. Two Mylar plate sealers.

***Note**: All kit components are stable until expiration date printed on the label. Do not use beyond expiration date.*

EQUIPMENT

- Microtiter plate reader capable of reading at a wavelength of 450 nm
- Microliter pipettes capable of accurately delivering 10 and 200 µL
- Wash bottle or plate washing system
- Distilled or deionized water
- One liter graduated cylinder
- Serological pipettes, 1, and 10 or 25 ml
- Disposable pipette tips
- Paper towels
- Timer

QUALITY CONTROL

1. The following control sera must be included in each analytical run:

   Negative Control
   IgG Cut-Off Calibrator
   Low Positive Control
   High Positive Control

2. Include a minimum of 1 blank well, containing sample diluent only for instrumentation calibration purposes.

3. If multiple plates are run, include the Cut-off calibrator and all three controls on each plate. It is recommended that until the user becomes familiar with the kit performance, all specimens, controls and Cut-Off Calibrator should be run in duplicate with the Cut-off Calibrator run twice for a total of four wells. If single wells are used, the Cut-off Calibrator should be run in triplicate.

4. Calculate the mean of the IgG cut-off calibrator determinations.

5. The mean OD value of the cut-off calibrator wells must be within 0.100-0.500 O.D. units. All replicate cut-off calibrator OD's should be within 0.10 absorbance units from the mean value.

   Report results as Index Values relative to the cut-off calibrator. To calculate Index Values, divide specimen optical density (OD) values by the mean of the cut-off calibrator absorbance values.

   A. The High Positive Control Index Values should be greater than 3.5.
   B. The Low Positive Control Index Values should be between 1.5 and 3.0.
   C. The Negative Control Index Values should be less than 0.8.

   If the above conditions are not met the test should be considered invalid and should be repeated.

QUALITY ASSURANCE

This procedure complies with the Quality Assurance Policy and implementing protocols of <INSERT NAME OF INSTITUTION>

PROCEDURE--STEPWISE

1. Set-up of the Assay:

   Remove the individual kit components from storage and allow them to warm to room temperature (20-25°C). Determine the total number of samples and controls to be tested. The high positive control, negative control, Cut-off Calibrator and a reagent blank must be included each time the assay is performed.

   Determine the number of microwells needed. After the strips and holder have warmed to room temperature, cut open the protective envelope and remove the plate containing the antigen coated microwell strips. Unused strips should be returned to the foil packet with desiccant and resealed completely. Store unused antigen wells at 2-8°C.

   Prepare the wash buffer according to the Preparation of Reagents section.

2. Serum Incubation:

   Prepare a 1:101 dilution of the high positive, low positive, negative, cut-off calibrator and each patient serum as follows:

   A. Add 10 µL of each sample to an appropriately labeled tube each containing 1mL of sample diluent.

   B. Soak test plate for 5 minutes with wash buffer, decant and tap vigorously to remove excess wash buffer. Blot the emptied antigen wells.

   C. Add 100 µL of sample diluent to a well as a reagent blank. Add 100µL of each diluted specimen, control or calibrator to appropriate wells.

   D. Cover the wells with a plate sealer and incubate the plate at room temperature (20-25°C) for 60 $\pm$ 1 minute.

   E. Wash the microwell strips 3X with 1X wash buffer.

      1) Vigorously shake out the liquid from the wells.

      2) Fill each well with wash buffer. make sure no air bubbles are trapped in the wells.

      3) Repeat steps (a) and (b) twice.

      4) Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the

wells. Visually inspect the plate to ensure that no residual wash solution remains.

3. Conjugate Incubation:

   A. Add 100 µL of conjugate solution to each well at the same rate and in the same order as the specimens were added.

   B. Cover the plate with the plate sealer provided and incubate at room temperature (20-25°C) for 30 ± 1 minutes.

   C. Wash the plate by following the procedure in step 2.E. (1) through (4).

5. Substrate Incubation:

   A. Add 100 µL of TMB substrate solution to each well at the same rate and in the same order as the conjugate was added (1 ml of TMB substrate is sufficient for 8 wells).

   B. Incubate the plate at room temperature (20-25°C) for 10 minutes ± 1 minute.

   C. Add 100 µL of stop solution to each well at the same rate and in the same order as the TMB substrate solution was added. Positive sample wells will turn from blue to yellow. After adding stop solution, tap the plate several times to ensure that the samples are thoroughly mixed.

   D. Set the microplate reader wavelength at 450 nm and measure the optical density (OD) of each well against the reagent blank. The plate should be read within 1 hour after addition of the stop solution.

CALCULATIONS

1. Cutoff OD Value

   To obtain the cutoff OD value, average the cut-off values.

2. Index Values

   Calculate an Index Value for each specimen by dividing its OD value by the mean cut-off OD from step 1.

REPORTING RESULTS

Reference Range: $\leq$0.90

Index Values are interpreted as follows:

Index Values	Interpretation
≤ 0.90	Negative
0.91 - 1.09	Equivocal
≥ 1.10	Positive

LIMITATIONS OF PROCEDURE

1. All results from this and other laboratory tests must be correlated with clinical history, epidemiological data, and other data available to the attending physician in making a diagnosis of HSV infection.

2. Patients with early HSV infection may test negative for IgG antibodies, since the IgG response to the gG antigen may be undetectable for up to 6 months post onset. Therefore, testing for IgM antibody is recommended. If a negative result is reported on a patient with signs and symptoms of HSV infection, repeat testing for IgG and IgM antibodies on a second sample obtained 1 to 6 months later is recommended.

Procedure: GUD PCR on the ABI 7900 HT Sequence Detection System

Prepared by	Date Adopted	Supersedes Procedure #
J. Hardick, P. Stamper, C.Gaydos	06-30-03	03-17-03

Review Date	Revision Date	Signature
06-30-03	06-30-03	

Distributed to	# of Copies	Distributed to	# of Copies

PRINCIPLE:

Multiple pathogens are reported to cause Genital Ulcer Disease (GUD): ***Treponema pallidum (TP),*** **Herpes Simplex Viruses 1 and 2 *(*HSV 1 & 2), and *Hemophilus. ducreyi (HD).***

Diagnosis in standard clinical laboratory for these pathogens is not always rapid or conclusive. Serology for syphilis has some inherent problems wih cross reactivity of epitope sites and is only indicative of exposure not of current infection. Darkfield examination for TP is not available except for sophisticated laboratories and culture is never performed because of the extremely fastidious nature of the *T.pallidum*. Culture for HSV1& 2 is possible in laboratories with the ability of tissue culture but is expensive and slow. Recent advance is HSV serology have made serologic testing feasible but it is not reliable for diagnosis of the ulcer, only of previous infection of HSV 1 & 2. *H. ducreyi* is difficult to culture and is fastitious and slow growing. This PCR method is an adaptation of the previously published PCR developed by Roche Molecular Diagnostics (Inianapolis, IN). (1). We have previously used this method for other studies (2, 3). The method is now adapted for real time PCR using Taqman technology for the ABI 7900 HT sequence detection system. (Foster City, CA).

SPECIMEN:

Patient Preparation: Clean the genital ulcer with a sterile swab moistened with saline.

Type of swab: Ulcer specimens should be collected using a dacron swab with a plastic shaft and stored dry in a sterile capped test tube at –70C until shipment to the laboratory.

Using the dacron swab, swab the ulcer thoroughly, paying special attention to the base (edges) of the ulcer. The prefered method is to store the swab in a dry condition. However, the swab can be thoroughly expressed into a suitable transport media (phosphate buffered saline or Roche M-4 Transport Media) When using a new transport media type, a validation must be done to insure that the transport media does not inhibit the PCR.

Handling Conditions: Uninoculated media should be refrigerated until use. Upon collection of the sample, it should be frozen immediately at -70° C unless testing is to occur with 24 hours.

PROCESSING UPON RECEIPT IN THE LABORATORY:

1. Thaw samples if necessary. Add 1 ml of sterile molecular grade water to the dry swab and vortex for 30 sec. [If samples were collected in a transport media, , thaw and vortec for 30 sec.]

2. Place 200 ul for extraction onto the MagNa Pure LC robot (Roche Diagnostic Systems, Indianapolis, IN). See Robot protocol. Use 20 ul of each extracted sample in the PCR reaction.

3. Include both positive and negative controls in the extraction process.

EQUIPMENT AND MATERIALS:

Equipment:
ABI 7900 HT Sequence Detection System

Materials:

PRIMER AND PROBE SEQUENCES:

T.pallidum **(47-kDa membrane immunogen gene)**

Primer K03A 5'GAAGTTTGTCCCAGTTGCGGTT3'

Primer K014 5'CAGAGCCATCAGCCCTTTTCA3'

Probe K017 5'TET-CGGGCTCTCCATGCTGCTTACCTTA-MGB3'

HSV 1 & 2 (Glycoprotein B gene)

Primer KS30 5'TTCAAGGCCACCATGTACTACAAAGACGT3'

Primer KS31 5'GCCGTAAAACGGGGACATGTACACAAAGT3'

Probe KS54 5'VIC-GGTCTCGTGGTCGTCCCGGTGAAA-MGB3'

***H. ducreyi* (16S rRNA gene)**

Primer K07A 5'CAAGTCGAACGGTAGCACGAAG3'

Primer K08A 5'TTCTGTGACTAACGTCAATCAATTTTG3'

Probe K015 5'FAM-CCGAAGGTCCCACCCTTTAATCCGA-MGB3'

Preparation:

MASTER MIX PREPARATION:

Reagent	Stock Concentration	100 reactions (ul)*	10 reactions (ul)*	1 reaction (ul)*
10x Qiagen HotstarTaq Polymerase Buffer	10X	1000 ul	100 ul	10ul
$MgCl_2$	25 mM	1200 ul	120 ul	12 ul
DNTPs	10 mM	200 ul each dNTP	20 ul each dNTP	2 ul each dNTP
Primers & Pro Bes	50 uM	50 ul each primer/probe	5 ul each primer/probe	0.5 ul each primer/probe
DH_20	NA	4200 ul	420 ul	42 ul
Q solution	NA	100 ul	10 ul	1 ul
Qiagen HotstarTaq Polymerase	NA	200 ul	20 ul	2ul

*-Reaction conditions are for a 100ul PCR reaction utilizing 80ul of master mix and 20ul of sample/template DNA.

Performance Parameters:

ABI 7900 HT PCR Reaction Conditions*:*

1. 50C, 2 min
2. 95C, 10 min
3. 40 cycles of: 95C, 30 sec; 62C, 30 sec; 72C, 30 sec
4. 72C, 20 min
5. 4C, hold

Storage Requirements:

Reagent	Storage Conditions
10x Qiagen HotstarTaq Polymerase Buffer	-70C
$MgCl_2$	-70C
DNTPs	-70C
Primers & Probes	-70C
dH_20	4C
Q solution	-20C
Qiagen HotstarTaq Polymerase	-70C
Samples	-70C

QUALITY CONTROL:

Controls positive control must include ***T.pallidum*, HSV 1 & 2, *H. ducreyi*** DNA or organism template.

1. Positive control for each pathogen. Use a low copy number concentration.
   a. *T.pallidum* template

   b. HSV 1 & 2 template
   c. *H. ducreyi* template
2. Negative control (sterile molecular water or carrier DNA such as salmon sperm DNA or one DNA primer.

The first use of a new transport media type must be extracted with positive control template using extraction method of choice to determine if the extraction process removes inhibitors to the PCR in the particular type of transport media.

PROCEDURE - STEPWISE:

1. Make the Master Mix for the samples in a "pre-amplification" biosafety laminar flow hood and dispense 80 ul to the appropriate wells.
2. Add the extracted DNA from the specimen (20 ul) to the appropriate well. Add the controls last. Use of a tray map is necessary for the location of samples on the plate. Seal plate with optical quality PCR caps (Applied Biosystems).

Performing a Run:

3. On the ABI 7900 computer, open the **SDS 2.0 icon** and select **open** from the file menu, and open the **GUD.sdt** ABI7900 template.
4. Highlight the wells on the 96 well plate that will be monitored where the reaction is to occur.
5. Ensure that all three detectors, FAM, TET and VIC have been assigned to the appropriate wells on the computer screen.
6. Save the run under **.SDS**, as a GUD run followed by the date (i.e., GUD_031703).
7. Once the run is saved, select the **instrument** tab on the right side of the screen. Under the instrument tag, select **open,** this should open the door of the instrument and allow the robotic arm to be loaded with a 96 well plate.
8. Load 96 well plate and select **start** from the instrument tab, and the run should start.
9. After run has successfully completed, select the **analysis** button (green arrow) to analyze the data acquired during the run.

CALCULATIONS:

Check for appropriate results from the controls in order to validate the run. Positive samples show significant gain of fluorescent signal for each organism both in the multicomponent view and the delta RN vs. cycle mode. (FAM: chancroid; TET: syphilis; and VIC: HSV). Gain of signal above the negative control background level for the particular fluor is indicative of the specific organism. Positive results are calculated by the equipment using as reference the crossing threshold (Ct) of the particular positive control..

SENSTIVITY AND SPECIFICITY:

The original reference (1) reported the following sensitivity and specificities:

	Sensitivity	Specificity	Reference method
TP:	91%	99%	Darkfield examination
HSV 1 & 2	100%	100%	Culture
HD	98.4%	99.6%	Culture

REPORTING RESULTS:

Reference Ranges:
Negative PCR results for each organism is normal. [Ct of 40].
Procedures for Abnormal Results:
Results are entered into the database as positive, negative, or inconclusive for each organism.
Reporting Format:
Positive, negative, or inconclusive for each of the 3 organisms.

PROCEDURE NOTES:

User should be familiar with ABI 7900 operation and maintenance.

LIMITATIONS OF THE PROCEDURE:

Limitation are based on the appropriateness and quality of DNA in the sample. Specimens can be checked by PCR for human beta globin to assess quality and amount of amplifiable human DNA if the quality of the integrity of the sample is questionable.

REFERENCES:

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2. Gadkari, D.A., T.C.Quinn, R.R. Gangakhedkar, S.M. Mehendale, A.D. Divekar, A.R. Risbud, K. Chan-Tack, M. Shephard, C. Gaydos, R.C. Bollinger. HIV-1-DNA shedding in genital ulcers and its associated risk factors in Pune, India. J. Acquir. Immuno-Def. Synd. Hum. Retrovir. 18:277-281, 1998.
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CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date: December 2002 Author: Ali Taylor, Patrick Chipaila
Title: Direct Wet Mount Microscopy/KOH Preparation		Revised Effective Date: Author: J. Westerman MT(ASCP)
Proc#: V.4.1-4	File Name: V.4.1-4 Wet Mount and KOH Preparation	

I. PURPOSE: To provide a procedure for the preparation, reading and microscopic examination of the direct wet mount and KOH preparation. These tests are useful for the detection of the presence of bacteria, fungi, and Trichomonas vaginalis. This SOP should enable the operating technologist to make a presumptive identification based on morphology and motility of the organisms.

II. MATERIALS AND EQUIPMENT:

Gloves
Glass slide with frosted end
Cover slips
Binocular microscope equipped with 10x and 40x objectives
Gram pen
Sterile physiological saline
10% K.O.H. (Potassium Hydroxide)
pH Paper
Plastic falcon tube with lid

NOTE: Concentrated KOH is a highly corrosive chemical that must be used with appropriate personal protective equipment including gloves and eye/face protection.

III. SPECIMEN TYPE AND STORAGE:

A. Specimen Type

1. Vaginal secretions obtained with a sterile swab during a pelvic examination. See the clinical Collection SOP for details on the collection.
2. **HPTN 035 –** Swabs are placed into tube with 6 drops of sterile physiological saline.
3. **HPTN 039 –** Samples are collected using transport swab with liquid stuarts media.
4. Collections are transported to the Satellite Laboratory within 30 minutes after collection.

B. Storage

1. To preserve the motility of T. vaginalis, the specimen should not be refrigerated and should be examined as soon as possible following collection.

IV. FORMS:

V.4.1A CIDRZ Wet Mount Bench Log
V.4.1B KOH Quality Control Log
V.4.1C Saline Quality Control Log
Satellite Laboratory Report Form

V. PROCEDURE:

A. Prepare Slides

1. Using a gram pen label two clean frosted ended slides with the specimen I.D.
2. Place a drop of saline on one slide and a drop of 10% KOH on the other slide.
3. Emulsify the swab on the saline slide and then the KOH slide.
4. Perform the "whiff" test by smelling the KOH slide **immediately** for a " fishy" amine odor.

B. Microscopic Evaluation at 10X and 40X

1. Cover the each slide (saline and KOH) with a cover slip and examine the slide preparation.
2. Examine the **saline** slide.
   a. Using the **10X** objective examine slide for the presence of:
      1) Epithelial cells
      2) Motile T. vaginalis
      3) Budding yeasts or pseudohyphae
   b. Using the **40X** objective examine the slide for:
      1) Clue cells using the following identifying criteria:
         a) Clue cells are epithelial cells covered with a variety of small gram positive and gram negative rods and curved rods.
         b) **Some times** the nucleus is occluded but **not always**.
         c) Look at the peripheral boarders of the epithelial cells for the fuzzy effect that is present when the bacteria are coating the surface.
      2) If clue cells are present, determine the number of clue cells (<20% or ≥20%).
         a) Count the total number of epithelial cells in the field.
         b) Count the total number of clue cells in the field.
         c) $\%\text{Clue cells} = \frac{\text{number of clue cells}}{\text{Total number of epithelial cells}} \times 100$
      3) If clue cells are NOT present, report <20%.
2. Examine the **KOH** slide.
   a. Using the **10X** and **40X** objectives examine the slide for the presence of:
      1) Yeasts
      2) Pseudohyphae

NOTE: Clue cells are epithelial cells that are covered with bacteria. Trichomonads are only seen in the saline preparation. They are lysed by KOH. Actively motile trichomonads are easily seen on low power objective (10X). The 40X objective is useful in the detection of less vigorous moving organisms when only the flagella may be moving.

C. Quality Control

1. 2nd Review:
   a. Once per month the laboratory supervisor or technologist will perform a 2nd read on a wet mount sample.
   b. The lab supervisor will work with the SRN to determine visits which have wet mount testing. The lab supervisor will organize a 2nd technologist or supervisor to go to the laboratory to perform the second read.
   c. The satellite technologist will not delay testing. Once the wet mount has been reported the sample will be saved for the second read.
   d. When wet mount testing is not routinely performed:
      1) The satellite laboratory technologies will call the laboratory protocol supervisor to request the second reading when a wet mount sample is received.
      2) The second read will be performed once per month. If no samples are received in a month, the second review is performed when samples are requested for testing.
   e. The second reading will be documented in the comment section of the patient/participant's results.
2. KOH:
   a. Prepare KOH using 10g of KOH into 100 ml deionized water.
   b. Assign lot number using the date of preparation followed by KOH. (example: 3OCT2005KOH) Write the lot number on the container.
   c. Expiry date is 6 months from preparation. Write the expiry date on the container.
   d. Weekly review for yeast or fungi. If seen discard and prepare new KOH solution. Document on **V.4.1B KOH Quality Control Log**.
   e. The pH is checked at preparation and monthly. Document on **V.4.1B KOH Quality Control Log**. A pH of $\geq$ 9 is acceptable.
2. Physiological Saline:
   a. Saline is prepared by the CIDRZ Central Laboratory:
      1) 8.5g of sodium chloride is added to 1 litre of distilled water.
      2) Weigh the sodium chloride, and transfer it to a clean leak proof autoclavable bottle marked to hold 1 litre.
      3) Add distilled water to the 1 litre mark, and mix until the salt is fully dissolved.
      4) Label the bottle and autoclave at 121 degrees celcius and dispense in to bottles for use.
      5) Culture saline.
      6) The solution is stable for six months.
      7) Discard if it becomes contaminated.
   b. Saline is cultured for bacteria.
   c. Saline is placed in sterile containers for distribution to the satellite laboratories. The lot number is the date of preparation. Write the lot number on the container. (example: 6OCT2005NaCl)
   d. The expiration date is 6 months from preparation. Write the expiry date on each container.
   e. Document quality control on **V.4.1C Saline Quality Control Log**.
   f. Saline tubes used by the clinical staff for **HPTN 035** are prepared in batches.
      1) Culture the saline prior to preparation of the saline collection tubes.

2) The results should be negative prior to distribution. If positive make new lot of saline.
3) Label each tube with the lot number and the expiry date.
4) Drop six drops of the sterile physiological saline into each tube and place the cap on.
5) Do not distribute the saline tubes until culture results are available.

3. Proficiency testing – CIDRZ laboratory participates in the review of slides on the HPTN website www.hptn.org every 6 months.

VI. RESULTS REPORTING:

A. Results are reported on **V.4.1A CIDRZ Wet Mount Bench Log**.

B. Results are then transcribed to the Satellite Laboratory Report form.
a. Positive or Negative for T.vaginalis.
b. Positive or Negative for Yeast.
c. Positive or Negative for clue cells.
1) Positive for "clue cells" is greater than or equal to 20% ($\geq$ 20%).
2) Negative for "clue cells" is less than 20% (<20%).
d. Positive or Negative for whiff test.

VII. LAB REFERENCE RANGE: NA

VIII. LIMITATIONS:

- Many intra-vaginal medications will leave oil drop lets that can make interpretation of direct wet mount difficult.
- There might be small and large forms of T. vaginalis with varying degrees of motility.
- Non motile trophozoites can be confused with white blood cells. This why diagnosis of T,vaginalis should be made upon finding motile ones. The errors in technique can decrease the sensitivity of wet mount detection of T.vaginalis:
- Incorrect collection of specimen from the end cervix.
- Use of cool saline.
- Delay in reading the smear.
- Contamination of the saline prep with KOH.
- Sloppy preparation with too much saline, causing organism to move rapidly across field.
- Thick wet mount preparation.

IX. SPECIAL NOTES: N/A

X. REFERENCES:

Sexually Transmitted Diseases, 3rd edition. (Eds) KK Holmes, Sparling F., Mardh P., Lemon SM, Stamm WE, Piot P, and J. Wasserheit. 1999. McGraw Hill, New York.

Cheesbrough, M. Medical Laboratory Manual for Tropical Countries. Volume 1 2nd ed. Butterworth – Heinemann Ltd. 1987: 560.

Wet Mount Bench Log

Test Date	PTID	Clue Cells (<20%, >20%)	Budding Yeast /Pseudohyphae	T. Vaginalis (Motile)	Tech 01 Initials/Code	Tech 02 Initials/Code	Comments

CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date: March 2001 Author: P. Chipaila, A. Taylor
Title: pH Paper Quality Control		Revised Effective Date: DRAFT Author: J. Westerman, P. Chipaila
Proc#: V.4.7-3	File Name: V.4.7-3 pH Paper Quality Control	

I. PURPOSE/PRINCIPLE: To provide a procedure for the quality control of pH paper using certified buffer solutions. It is the policy of the HPTN Central Laboratory to run positive and negative quality control on each lot number of pH paper prior to use for patient care. Any lot that does not pass the quality control will not be used

II. MATERIALS AND EQUIPMENT:

pH paper (3.6 – 6.1) Allegience
Control Buffer solutions Certified Buffer solutions, pH 4.0 and pH 6.0
(Fisher order # for pH 4 is SB99-500)
(Fisher order # for pH 6 is SB106-500)

III. SPECIMEN TYPE / STORAGE:

pH Strips are stored at room temperature 15 - 30°C
Buffer solutions of pH 4.0 and pH 6.0 are stored at room temperature 15 – 30 °C.

IV. FORMS:

V.4.7A pH Paper Quality Control Form

V. PROCEDURE:

A. <u>Lot number and Expiry date of pH paper</u>

1. Assign lot number to the pH strips using the receive date (ie. 26Sept2005).
2. The lot number will be written on each container
3. The expiration date of one year will be written on each container.

B. <u>pH Test</u>

1. pH quality control is performed on new lot numbers as received by the central laboratory prior to distribution to the clinics.
2. Remove 2 pH strips from the container
3. Add one drop of pH 4 buffer solution to one strip and one drop of pH 6 to the other strip.
4. Compare the color on the strips to the chart on the container.
5. Write the results of the pH test on the pH Paper Quality Control form.
6. Record the lot# and the expiry date on the QC form.
7. Record the lot number and expiry dates of the certified buffers on the QC form.

VI. RESULTS/REPORTING:

A. If the colors match to within one color category to the corresponding pH on the chart, the test passes.
B. If one or both of the pH buffers does not match, repeat the test.
C. If on repeat the colors still do not match the control chart,
1. Use a different pH strip.
2. If the new strip gives the same results use a new buffer solution.
3. If the test still fails, test another container of buffer and discard any containers that fail the test. The buffers have a color indicator that will turn when the buffer is no longer good.

D. Report any failures to the supervisor.
E. Document any problems with the strips or buffers in the comment section at the bottom of the QC record.

VII. LAB REFERENCE RANGE: N/A

VIII. LIMITATIONS: N/A

IX. SPECIAL NOTES:

- QC on the pH strips is performed by the HPTN Laboratory Coordinators at CIDRZ Central Laboratory prior to distribution.
- pH strips are sent out to the clinics for use by the nurses.
- HPTN Laboratory technologists will perform quarterly QC assessments of the pH strips after strips are sent to the clinics.

X. REFERENCES:

024 Specific Laboratory Operations Manual

Magee-Womens Research Institute, Reproductive Infectious Disease Research Laboratory, Pittsburgh, Pa.

pH Paper Quality Control Log

pH Buffer 4.0 Lot#_____ pH Buffer 4.0 Expiry Date_____

pH Buffer 6.0 Lot#_____ pH Buffer 6.0 Expiry Date_____

Date of Testing	pH Paper Lot #	pH Paper Expiry Date	pH of Buffer 4.0	pH of Buffer 6.0	Pass/Fail	Tech Initials/ Code	Tech Initials/ Code	Comments

Quality Control is performed quarterly and with each shipment/new lot number

Procedure: Becton Dickenson RPR Card Test

Prepared by	Date Adopted	Supersedes Procedure #
Jeffrey Yuenger		

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

PRINCIPLE:

RPR Card antigen suspension is a carbon particle cardiolipin antigen that detects "reagin", an antibody-like substance present in sera from syphilitic persons, and occasionally in sera of persons with other acute or chronic conditions. This is a screening test and not a definitive test for syphilis; all positive sera must be confirmed by another method. When a specimen contains antibody, flocculation occurs in the presence of RPR antigen reagent. The coagglutination of the carbon particles in the RPR antigen reagent and REACTIVE sera appear as black clumps (similar to flecks of pepper) against the white background of the plastic-coated card. This coagglutination is read macroscopically. By contrast, NONREACTIVE sera appear to have an even light gray color.

SPECIMEN:

Patient Preparation:

No special preparation of the patient is required. Sera may be drawn form either fasting or non-fasting individuals.

Type:

Human Sera.
NOTE: Gross lipemia or marked hemolysis will obscure reading the agglutination reaction, so avoid those sera and request another blood specimen be collected.

Handling Conditions:

Collect blood by routine venipuncture into a clean, dry tube without anticoagulant and allow blood to clot. Clotting may be accelerated by placing the blood into a 37º C incubator or heating block. Centrifuge the blood tube at a force sufficient to sediment cellular elements. Keep the serum to be tested in the original collection tube, which will eliminate errors due to mislabeled transfer tubes. Plasma from EDTA tubes may be used for RPR testing if the plasma is less than 48 hours old.
NOTE: Handle all sera and blood tubes as if they are capable of transmitting blood-borne viruses (e.g. Hepatitis B or C, and HIV).

EQUIPMENT AND MATERIALS:

Equipment:

Rotator, 100 $\pm$ 2 rpm, circumscribing a circle of 2 cm in diameter, with automatic timer, friction drive, and a cover containing a moistened blotter or sponge.

Materials:

1. RPR Card Antigen Suspension, a reagent component part of the Macro-Vue RPR Card test kits.
2. Dispensing bottle supplied with each kit.

Additional Materials needed but not supplied:

1. One of the following Macro-Vue RPR Card Test Kits: 18mm Circle Test – Kit No. 104, 110, 115 (qualitative) or No. 112 (quantitative).
   Teardrop Test: Kit No. 100 (hand rotation)
2. Control sera with established patterns of graded reactivity that should be included in each day's testing to confirm optimal reactivity of the antigen suspension. Macro-Vue RPR Card Test Control Cards may be used to test the reactivity of the antigen suspension.
3. Saline (0.9%) for use in quantitative testing.
4. Serum nonreactive to syphilis in 0.9% Saline, required for diluting test specimens giving a Reactive result at the 1:16 dilution.
5. Dispenstirs or pipettors to dispense 50µl aliquots.

Preparation:

Prior to opening a vial of antigen, vigorously shake the ampule for 10 to 15 seconds to suspend the antigen/charcoal mixture and disperse any carbon particles that may have become lodged in the neck of the ampule. If any carbon should remain in the neck of the ampule after this shaking, no additional effort should be made to dislodge it, as this will only tend to produce a coarse antigen.
Attach the needle to the tapered fitting on the plastic dispensing bottle. Be sure the antigen is below the breakline; snap the ampule neck and withdraw all of the antigen into the

dispensing bottle by collapsing the bottle and using it as a suction device. Shake the card antigen-dispensing bottle gently before each series of antigen drops.

Performance Parameters:

Each RPR Card Antigen suspension lot number is tested for the established pattern of reactivity against reference antigen suspensions by the manufacturer and meets the Centers for Disease Control (CDC) product specifications for performing the RPR 18mm Card Test. These performance characteristics were established from a large number of papers that have appeared in the scientific literature, from routine daily test performance in syphilis serology test laboratories, and are in conformity with CDC specification.
Reported studies show the RPR Card Test has adequate sensitivity and specificity in relation to clinical diagnosis and a reactivity level similar to that of the VDRL slide test.

Storage Requirements:

Refrigeration (2-8º C) is required for the RPR Card Test Antigen suspension.

CALIBRATION:

Standard Preparation:

None

Calibration Procedure:

None

QUALITY CONTROL:

3. Controls with established patterns of graded reactivity should be included in each days test run to confirm optimal reactivity of the antigen suspension. This Laboratory uses the RPR Quality Control Cards, which have the sera dried on the card, supplied with the RPR Antigen as part of the Quality Control Program.
4. On the day tests are performed, the antigen suspension should be checked with controls of graded reactivity using the particular test procedure. Only those antigen suspensions that give th3 prescribed reactions should be used. Control cards, RPR Antigen suspension, and test specimens should be at room temperature (23-29º C) before being used.
5. The Laboratory uses prepared quality control cards (RPR Card Test Control Card), which contain dried serum in an 18mm circle. The cards contain sera that are NONREACTIVE, REACTIVE, and MINIMALLY REACTIVE. Reconstitute the dried sera portions, with 50 µl of distilled water, and spread the water/serum mixture to the periphery of the 18 mm circle. After the sera are reconstituted, test with RPR Antigen in the same manner as a patient specimen.

6. Check delivery of the needle by placing the needle firmly on a 1 ml pipette, fill the pipette with antigen suspension, and holding the pipette in a vertical position, count the number of drops delivered in 5 ml. The acceptable number of drops of antigen in 5 ml is 29-31 drops. If the needle does not give the acceptable number of drops, discard it and use another 20 gauge (yellow hub) needle.

PROCEDURE - STEPWISE:

The test kit includes Dispenstirs to assist in transferring serum samples. (An alternative to using Dispenstirs is to pipet 50µl of serum with a regular laboratory pipette).

1. Hold the Dispenstir device between thumb and forefinger near the stirring (sealed) end. Squeeze the Dispenstir and do not release pressure until open end is below surface of unheated serum, holding the specimen tube vertically to minimize stirring up of the cellular elements when using the original blood tube. Release finger pressure to draw up the sample.
2. Holding the Dispenstir in a vertical position directly over the card test area to which the specimen is to be delivered (not touching card surface), squeeze the Dispenstir device allowing one drop of specimen to fall onto the card. The amount delivered by the Dispenstir is approximately 50 µl; Dispenstirs are designed to expel slightly in excess of 50 µl to compensate for the small amount of specimen retained by the stirring end.
3. Invert the Dispenstir device and with the sealed stirring end, spread the specimen filling the entire surface of the 18 mm circle.
4. Gently shake the antigen dispensing bottle before use. Holding in a vertical position, dispense several drops onto the corner of the RPR test card to make sure the needle passage is clear. Holding the needle one inch above and perpendicular to the card, place one "free falling" drop (through the 20 gauge yellow hub needle) onto each test area. DO NOT STIR! Mixing of antigen and serum to be tested is accomplished during the rotation. Pick up the pre-dropped antigen from the card.
5. Rotate the card for eight (8) minutes, under a humidifying cover on a mechanical rotator set for 100 rpm.

CALCULATIONS:

This test if verified by visual confirmation. No calculations are needed.

REPORTING RESULTS:

Following rotation, to help differentiate NONREACTIVE from MINIMALLY REACTIVE sera, a brief rotation and tilting of the card by hand (3 or 4 to and fro motions) must be made. Immediately read macroscopically in the "wet" state under a high intensity incandescent lamp or strong daylight.

Report as: REACTIVE: Shows characteristic clumping ranging form slight but definite (minimal to moderate) to marked and intense.

NONREACTIVE: Shows a slight roughness or no clumping.

NOTE: There are only two possible test outcomes with the RPR Card Test: REACTIVE or NONREACTIVE, regardless of the degree of reactivity. REACTIVE minimally to moderate, showing slight but definite clumping, is always reported as REACTIVE.

All reactive results should be confirmed by retesting the specimen using a quantitative procedure to determine the level of antibody. The REACTIVE specimen should also be referred for confirmatory syphilis testing.

Reference Ranges:

Sear from individuals who have not been exposed to the treponeme T. pallidum will test will test NONREACTIVE by the RPR Card Test.

With cardiolipin type antigens, biological false positive (BFP) reactions have been reported in diseases such as infectious mononucleosis, leprosy, malaria, lupus erythematorsus, vaccinia and viral pneumonia

Procedures for Abnormal Results:

Quantitative Assay of REACTIVE Specimens:

1. For each serum specimen to be tested, place 50 µl of 0.9% saline into circles numbered 2 to 5 on a RPR Test Card. A dispenstir or pipette may be used. DO NOT SPREAD THE SALINE!
2. Using a pipette, place 50 µl of serum to be tested in circle 1 and into the saline drop in circle 2.
3. Change pipette tips and transfer 50 µl of serum/saline from circle 2 to circle 3, take 50 µl of serum/saline from circle 3 and place into circle 4, take 50 µl of serum/saline from circle 4 and place into circle 5. From circle 5 discard 50 µl of the serum/saline mixture. At this point, each circle contains 50 µl of liquid.
4. Using a new stirrer (broad end) for each specimen, start at the highest dilution of serum (circle 5) and spread serum/saline mixture, filling the entire surface of the circle. Proceed to circles 4,3,2, and 1 to accomplish similar spreading.
5. Gently shake antigen dispensing bottle before use. Holding in vertical position, dispense several drops on the corner of the test card. Then place one “free falling” drop onto each test area. DO NOT RESTIR! Mixing of antigen with serum/saline dilutions will be accomplished during the rotation. Pick up pre-dropped antigen from edge of test card.
6. Rotate for eight (8) minutes under a humidifying cover on a mechanical rotator with a speed set at 100 rpm.

Following rotation, to help differentiate NONREACTIVE from REACTIVE results, a brief rotation and tilting of the card by hand must be made. Then immediately read macroscopically in the “wet” state under a high intensity incandescent lamp or strong daylight.

Reporting Format:

Report in terms of the highest dilution giving a REACTIVE reading to minimal and moderate reactions.

Examples:

1:1 Circle 1	1:2 Circle 2	1:4 Circle 3	1:8 Circle 4	1:16 Circle 5	Report as:
5.Rm	N	N	N	N	REACTIVE, 1:1 dilution
R	R	R	N	N	REACTIVE, 1:4 dilution
R	R	R	R	N	REACTIVE, 1:8 dilution

If the highest dilution test (1:16) is REACTIVE, proceed as follows:

1. Prepare a 1:50 dilutions of NONREACTIVE serum in 0.9% saline. (This diluent is to be used for making dilutions beyond 1:16)
2. Prepare a 1:16 dilution of the test serum by adding 100 µl of serum to 1.5 ml of 0.9% saline. Mix thoroughly.
3. Place 50 µl of the 1:50 NONREACTIVE serum diluent in circles 2,3,4, and 5.
4. Using a pipette, place 50 µl of the 1:16 dilution of test specimen in circle 1.
5. Refill pipette, make serial two-fold dilutions and complete tests as described under steps 3 to 6 of Quantitative Assay of REACTIVE specimens.

The dilutions of this test are as followed: (Circle 1 to Circle 5) 1:32, 1:64, 1:128, 1:256, and 1:512.

Higher dilutions are prepared if necessary in 1:50 NONREACTIVE serum not 0.9% saline.

PROCEDURE NOTES:

Each RPR Card Antigen suspensions lot number is tested for the established pattern of reactivity against reference antigen suspensions by the manufacturer and meets the Centers for Disease Control (CDC product specifications for performing the RPR 18 mm Card Test. These performance characteristics were established form a large number of papers that have appeared in the scientific literature, from routine daily test performance in syphilis serology test laboratories, and are in conformity with CDC specification.

Reported studies show the SPS Card Test has adequate sensitivity and specificity in relation to clinical diagnosis and a reactivity level similar to that of the VDRL slide test.

LIMITATIONS OF THE PROCEDURE:

The diagnosis of syphilis should not be made on a single REACTIVE result without the support of a positive history or clinical evidence. Therefore, as with any serological testing procedure, Reactive Card test specimens should be subjected to further serologic studies to confirm the

initial reaction. Specimens that are REACTIVE in qualitative testing should be quantitated to establish a baseline form which changes in titer can be determined, particularly for evaluating the effectiveness of treatment. It is also desirable to quantitative specimens that are NONREACTIVE-ROUGH so that an infrequent zonal specimen may be revealed.

REFERENCES:

1. Macro-Vue RPR Card Test Product Insert; Hyson, Wescott, and Dunning; 1981.
2. Manual of Test for Syphilis, PHS Publication 411; 1969

Procedure: Serodia-TP.*PA* Reagents for the Detection of Antibodies to *Treponema pallidum*

Prepared by	Date Adopted	Supersedes Procedure #
Jeffrey D. Yuenger		

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

PRINCIPLE:

The Serodia-TP-PA test is based on the agglutination of colored gelatin particle carriers sensitized with *T. Pallidum* (Nichols stain) antigen. Serum or plasma samples are serially diluted in Sample diuluent in microplate wells. Sensitized Gelatin Particles are added to respective wells and the contents of the plate mixed by hand or on a tray mixer. The mixture is incubated stationary for 2 hours at room temperature. Serum or plasma containing specific antibodies will react with the antigen-sensitized colored gelatin particles to form a smooth mat of agglutinated particles in the microtitration tray. A compact button formed by the settling on the non-agglutinated particles characterizes negative reactions. The test is designed to be used exclusively with microtitration techniques. The agglutination patterns and interpretation of the test are clear cut and easy to read visually or with the aid of a tray viewer.

SPECIMEN:

Patient Preparation:

Specimens containing erythrocytes or other visible matter should be centrifuged prior to testing to prevent interference with test results.

Type:

Human serum or EDTA, Sodium Citrate, or Heparin plasma may be used

Handling Conditions:

Store patient serum samples in a refrigerator at 2-8Cº if testing is done within 5 days. Sera may be frozen and thawed only once. Heat inactivation is not necessary for the patient sera. However, previously heat treated sera may be used. Do not inactivate or freeze plasma and use within 48 hours.

EQUIPMENT AND MATERIALS:

Equipment:

Microtiter plate mixer (optional)
Microtiter plate viewer (optional)

Materials:

Materials supplied:
The kit contains sufficient reagents to perform 100 or 220 qualitiative tests. Each kit contains the following reagents and accessories:

1. Reconstituting Solution (8ml for 100 assay kit and 18ml for 220 assay kit): Aqueous solution of 0.05M Phosphate Buffer containing 0.2M NaCl, 0.6% normal rabbit serum, and 0.06% sodium azide, at pH 7.00 – 7.60. The solution is used for reconstituing the Sensitized and Unsensitized Particles.
2. Sample Diluent (29ml for 100 assay kit and 60ml for 220 assay kit): The solution is used for diluting human specimens in the assay. Aqueous solution of 0.05M Phosphate Buffer containing 0.9M NaCl, 2% normal rabbit serum, 0.1% rabbit testicular extract, and 0.1% sodium azide at pH 6.70 – 7.30.
3. Sensitized Particles (five 0.6ml vials for 100 assay kit and four 1.5ml vials for 220 assay kits): Lyophilized preparation of colored gelatin particles sensitized with *Treponema pallidum* antigen. At the time of use, add the Reconstituting Solutions to the volume indicated on the Particles vial label and in Table 1 above. The rehydrated reagent contains a 1% suspension of sensitized gelatin particles and 0.8% (w/v) soduim azide as preservative.
4. Unsensitzed Particles (five 0.6ml vials for 100 assay kits and four 1.5ml vials for 220 assay kits): Lyophilized preparation of colored gelatin particles. At the time of use, add the Reconstituting Solution to the volume indicated on the Particles vial label and in Table 1 above. The rehydrated solution contains a 1% suspension of unsensitized gelatin particles and 0.8%(w/v) sodium azide as preservative.
5. Positive (reactive) control serum (0.5ml vial for 100 assay kits and 220 assay kits): This liquid serum containing rabbit antibodies to *T. pallidum* should demonstrate a titer of 1:320 final dilution, ± one doubling dilution, when tested according to the procedure described below. Control contains 0.099% sodium azide as preservative.
6. Dropper: 2 pcs. In 100 and 220 Test Kits – To dispense approximately 25 µl per well. One dropper to be used exclusively for dispensing reconstitued Sensitized Particles and the other dropper for dispensing the Unsensitized Particles.

7. Non-reactive Control serum: 1 vial containing 0.5 ml human serum with 0.1% sodium azide as preservative.

Materials not supplied:
1. Polystyrene microplate with "U" shaped wells. Plates should be free from dust, lint, and scratches.
2. Micro-pipettor with tips – to dispense 25µl and 100 µl – for dispensing and diluting serum samples.
3. Pipettes – 1.0 ml serological for reconstitution.

Preparation:

1. Reconstituting Solution, Sample Diluent and Positive (Reactive) Control are liquids ready for use and require no reconstitution.
2. Sensitized Particles and Unsensitized Particles must be reconstituted with the Reconstituting Solution using the volume listed on the vials (see Table 1). Once opened, dispense the appropriate amount of Reconstituting Solution. Mix the reconstituted reagents thoroughly and allow them to stand at room temperature for at least 30 minutes prior to use. Mix particles again prior to dispensing. When the particles are reconstituted, write the date reconstituted plus 7 days out-date and technicians initials on the label. The particles should not be used past this 7 day reconstituted date.

Performance Parameters: Expected Results

Studies performed on 175 normal male and female and 171 normal pregnant female populations indicated a reactive rate of 2.6% (9/346) from people located in the Southeast United States. Six of these donors were also reactive on RPR testing. The population ranged in age from 14 to 40 years of age. The rate of reactivity will vary according to geographic location and social demographics of the population.
One hundred percent (100%) of samples tested from both treated and untreated patients in both the primary and secondary stages of the disease were detected by the Serodia TP-PA test. Samples from individuals with autoimmune diseases, Toxoplasmosis, *H. pylori*, IV drug users, and HIV demonstrated an 11.4% (18/158) reactive rate. These same samples were also reactive by the MHA-TP hemagglutination assay.

Storage Requirements:

Store all reagents at 2-10ºC both before and after opening or reconstitution. DO NOT FREEZE. Reconstituted Sensitized and Unsensitized Particles should be used within 7 days. Liquid reagents are stable through the labeled expiration date. Do not use reagents after the expiration date marked on the kit.

CALIBRATION:

Standard Preparation:

none

Calibration Procedure:

none

QUALITY CONTROL:

1. The Positive (Reactive) Control should be processed at least once on the day of testing or when a batch of specimens is run and should yield a positive reaction. The Positive Control Serum may be titered to confirm the 1:320 endpoint, ± one doubling dilution, as additional quality control for the assay. To titer the Control, follow the Titration Procedure described below.
2. A Non-Reactive Control should be run at least once on the day of testing or when a batch of specimens is run and should yield a negative reaction. A separate Non-Reactive Control is provided with the kit or an in-house specimen can be used. If the Non-Reactive Control is not with the kit or more is needed, the control can be obtained from the Technical Service Department.
3. Confirm that the reaction with Unsensitized Particles (1:40 final dilution) is Negative (-) for each patient sample.
4. When titering the Positive (Reactive) Control Serum, confirm that the titer of the Positive (Reactive) Control Serum is 1:320 ± one doubling dilution, (final dilution) for the Sensitized Particles for each assay run (see Table 3)
5. If an assay does not meet Quality Control parameters listed above, the patient results from that assay should not be reported.

Titration Procedure for Positive (Reactive) Control (See Table 3)

1. Use a "U" shaped microplate sideways. One row (12 wells) is necessary to test one Positive (Reactive) Control dilution series.
2. Place 4 drops (100 µl) of Sample Diluent into Well #1 and 1 drop (25 µl) into Wells #2 through #12.
3. Using a micropipette, add 25 µl of Positive (Reactive) Control Serum into Well #1. Mix by filling and discharging the micropipette 5 or 6 times with the solution in Well #1. Then transfer 25 µl of the diluted solution in Well #1 with a micropipette and transfer it into Well #2. Mix well again and transfer 25 µl into Well #3. Repeat mixing and transfer respectively through Well #12 to obtain serial doubling dilutions. Discard the 25 µl of the solution remaining in the pipette after mixing Well #12.
4. Place 1 drop (25 µl) of the Unsensitized Particles into Well #3 and 1 drop (25 µl) of the Sensitized Particles into Wells #4 through #12 using the droppers supplied in the kit.
5. Mix the contents of the wells thoroughly (for approzimately 30 seconds) using a plate mixer (automatic vibratory shaker). DO NOT USE A ROTATOR. Then cover the plate

with an empty plate or microplate cover and place it on a level surface. Allow it to stand at room temperature (15-30°C) for 2 hours before reading. The incubation can be extended to overnight without any perceptible differences in patterns.

Table 3

Well #	1	2	3	4	5	6	7	12
Sample Dilutent(µl): Positive (Reactive) Control (µl):	100 + 25	25 + 25	25 + 25	25 + 25	25 + 25	25 + 25	25 + 25	25 25 *
Dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:10,240
Unsesitized Particles (µl):			25					
Sensitized Particles (µl):				25	25	25	25	25
Final Dilution			1:40	1:80	1:160	1:320	1:640	1:20,480
Mix, cover plate, and incubate for 2 hours								
Interpretation								

* Discard

1.PROCEDURE - STEPWISE: See Table 2

Four wells are required for each patient sample and Control(s) run in this assay. Well #1 & 2 are for dilution of sample. Well #3 for Unsensitized Particles and Well #4 for Sensitized Particles. The Positive (Reactive) and Non-Reactive Controls should be included in each run.

1. Place 4 drops (100 µl) of Sample Diluent in Well #1, and 1 drop (25 µl) in Wells #2 - #4 using a calibrated pipette dropper.
2. Using a micropipette, add 25 µl of patient specimen or Positive or Non-Reactive Control Sera into Wells #1.
3. Mix the contents of Well #1 by filling and discharging the micropipette 5 or 6 times. Then, using the micropipette, transfer 25 µl of the diluted solution from Well #1 into Well #2. Mix the contents of Well #2 in the same manner as stated above and transfer 25 µl into Well #3. Following the same procedure, mix the contents of Well #3 and transfer 25 µl into Well #4, mix and discard the 25 µl of solution remaining in the pipette after mixing Well #4.
4. Place 1 drop (25 µl) of Unsensitized Particles in Well #3, and 1 drop (25 µl) of Sensitized Particles in well #4 using the droppers supplied in the kit.
5. Mix the contents of the wells thoroughly (for approximately 30 seconds) using a plate mixer (automatic vibratory shaker). DO NOT USE A ROTATOR. Then cover the plate with an empty plate or microplate cover, and let stand at room temperature (15-30°C) for 2

hours before reading. The incubation can be extended to overnight without any perceptible differences in patterns.

Table 2

Well #	1	2	3	4
Sample Diluent (µl) Specimen Diluent (µl)	100 + 25	100 + 25	100 + 25	100 + 25discard
Specimen Dilution	1:5	1:10	1:20	1:40
Unsensitized Particles (µl)			25	
Sensitized Particles (µl)				25
Free Dilution			1:40	1:80
Mix, cover plate, and Incubate for 2 hours				
Interpretation				

REPORTING RESULTS:

Reference Ranges: Interpretation of Results

Place the plate onto a flat surface, preferably with a white background, and visually observe the pattern of agglutination in each will as described below. Note: A plate viewer may be used to aid in enhancing the visual interpretation.
Carefully place the microplate on a plate viewer (with indeirect lighting), and read the agglutination patterns of the Unsensitzed and Sensitized Particles for each specimen and Control. Readings are scored usingt the criteria described in Table 4 below:

Table 4

Settling Patterns of Particles	Reading	Interpretation
Particles are concentrated in the shape of a button at the center of the well with a smooth round outer margin.	(-)	Non Reactive
Particles are concentrated in the shape of a compact ring with a small hole in the center and a smooth outer margin.	(-)	Non Reactive
Particles are concentrated in the shape of a compact ring with a hole in the center and a smooth outer margin.	($\pm$)	Non Reactive
Particles for a large ring with a rough multifor outer margin and peripheral agglutination, surrounding a small red circle.	(1+)	Reactive
Agglutinated particles spread out covering the bottom of the well uniformly, surrounded by a red circle.	(2+)	Reactive

Smooth mate of cells covering less than the entire bottom of the well, and may be surrounded by a faint ring.	(3+)	Reactive
Smooth mat of cells covering the entire bottom of the well, edges sometimes folded.	(4+)	Reactive

Procedures for Abnormal Results: ABSORPTION PROCEDURE

6.In most cases, test samples do not show agglutination with Unsensitized Particles. However, if a test sample produces agglutination with both Unsensitized and Sensitized Particles, it should be re-tested after using the following absorption procedure:

1. Place 0.95 ml of reconstituted Unsensitized Particles in a small est tube.
2. Add 50 µl of test speciemen and mix thoroughly using tube mixer and incubate at room temperature for 20 – 30 minutes (mix manually 1 or 2 times)
3. Centrifuge for 5 minutes at 2,000 r.p.m. Place 50 µl of supernatant (absorbed at a 1:20 ratio of test specimen to Unsensitized Particles) to Well #3 of the microplate.
4. Add 1 drop (25 µl) of Sample Diluent into Well #4 of the microplate using a micropipette, transfer 25 µl of Well #3 (absorbed 1:20 diluted sample) into Well #4. Mix completely by filling and discharging the micropipette 3 or 4 times with fluid in Well #4, in order to make a doubling dilution, and then discard the 25 µl of the solution remaining in the pipette after mixing.
5. Place 1 drop (25 µl) of Unsensitized Particles in Well #3 and 1 drop (25 µl) of Sensitized Particles in Well #4.
6. Follow the original procedure and read the patterns.

Reporting Format:

REACTIVE: A specimen showing Non-Reactive with Unsensitized Particles (final dilution 1:40) but demonstrating a reaction of 1+ or more at any dilution 1:80 or over with Sensitized Particles is interpreted as Reactive in this test. The endpiont titer is determined as the highest serum dilution showing a 1+ or greater reactive pattern. If a serum sample demonstrates a positive reaction with <u>both</u> the Sensitized and Unsensitzed Particles, retest using the Absorption Procedure described above. A reactive treponemal test indicates past or present infection and usually remains reactive for life.

INCONCLUSIVE/INDETERMINATE: A specimen showing Non-Reactive with Unsensitized Particles (final dilution 1:40) but demonstrating a plus/minus reaction with Sensitized Particles at a 1:80 final dilution, is regarded as inconclusive or indeterminate. In such cases, it is recommended that the assay be retested, or another sample drawn and assayed in two weeks. The result of the repeated test should be reported if found to be Reactive or Non-Reactive. Repeated inconclusive results should be reported as Inconclusive for further follow-up and/or confirmed by other methods such as FTA-ABS.

NON-REACTIVE: Regardless of the reaction pattern with Unsensitized Particles, a specimen showing Non-Reactive with Sensitized Particles at a 1:80 final dilution is

regarded as Non-Reactive in this test. A Non-Reactive result indicates no past or present infection, but during incubating-stage syphilis, a non-reactive result may also occur.

PROCEDURE NOTES:

LIMITATIONS OF THE PROCEDURE:

1. The Serodia TP-PA test is specific for detecting *Treponema pallidum* antibodies in serum or plasma samples. It does not detect *T. Pallidum* directly.
2. As with all serological tests for syphilis, interpretation of results obtained with the Serodia TP-PA Syphilis Antibody test must be used in conjunction with the patients clinical symptoms, medical history and other clinical and/or laboratory findings to produce an overall clinical diagnosis.
3. Specimens giving inconclusive results in the assay should be re-tested. A repeated inconclusive specimen should be reported as Inconclusive for follow-up and another specimen drawn in two weeks for testing and/or confirmed by other methods, such as FTA-ABS.
4. The Serodia TP-PA is less sensitive than the fluorescent treponemal antibody absorption (FTA-ABS) test in untreated primary syphilis but compares favorably in all other stages of syphilis.
5. All treponemal tests tend to remain reactive following treponemal infection; therefore, they should not be used to evaluate response to therapy. Because of the persistence of reactivity, probably for the life of the patient, the treponemal tests are of no value to the clinician in determining relapse or re-infection in a patient who has had a reactive Serodia TP-PA result.
6. The Serodia TP-PA may be reactive in a small percentage (less than 1%) of normal or healthy persons; these false-positive results are often transient, their cause unknown. False positive Serodia TP-PA results may occur in association with other underlying illnesses.
7. The Serodia TP-PA may be reactive in persons from areas where yaws or pinta was, or is, endemic.
8. The Serodia TP-PA, as do all laboratory tests,performs best in populations at risk for the disease for which the test has been developed.
9. Samples from patients with HIV, leprosy, Toxoplasmosis, *H. pylori*, and drug addiction may react, on occasion, with either the sensitized or the unsensitized particles, causing false-positive or inconclusive results.

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Procedure: InPouch TV *Trichomonas vaginalis* Test

Prepared by	Date Adopted	Supersedes Procedure #
Denise L. McNairn	May 11, 2000	N/A

Review Date	Revision Date	Signature

Distributed to	# of Copies	Distributed to	# of Copies

SUMMARY AND PRINCIPLE:

Trichomoniasis is a sexually transmitted infection caused by the flagellated protozoan, *Trichomonas vaginalis*. It is recognized as one of the most prevalent forms of sexually transmitted infections (STI) world-wide, in both males and females.[1] Trichomoniasis may result in premature rupture of membranes in pregnancy and can also cause nongonococcal urethritis in males.[2,3]

In InPouch TV, *Trichomonas vaginalis*, test is a self-contained system for the detection of *T. vaginalis* from female vaginal samples or male urine samples. The proprietary medium is selective for the transport and growth of *T. vaginalis*, while inhibiting the growth of contaminating microorganisms, which might interfere with a reliable diagnosis.

The InPouch consists of a high-barrier plastic pouch with two V-shaped chambers connected by a narrow passage. The two-chamber system permits direct observation (wet-mount of a newly collected specimen in the upper chamber before expressing the inoculum into the lower chamber for culture incubation.

The sample can be concentrated by letting the cellular materials settle to the bottom of the chamber before observation. Microscopic observation of the chamber is done using a plastic microscope viewing clip.

An inoculum containing 1 to 10 organisms is sufficient to cause a positive test.[4] Transport and off-site testing are performed easily due to the flexible packaging and integral design of the InPouch.

SPECIMEN:

Patient Preparation:

Type:

Vaginal: Specimen collected from posterior fornix using a cotton or Dacron swab with either a wooden or plastic sticks are acceptable.

Self-administered vaginal specimen collection: Ask the patient to insert the sterile cotton swab an inch or two into her vagina. After swabbing, using a rotational motion, the patient should remove the swab and hand the swab to the laboratory technician or nurse.

Male Urine: Urine (15ml) is collected in a clean screw-cap collection container; specimen is centrifuged for sedimentation for 5 minutes at 500 rpm.

Handling Conditions:

After inoculation into the InPouch, the specimen should be maintained between 18-37º C while awaiting culture. We recommend culturing any specimen the day of sampling, although specimens have maintained trichomonad viability for 24 hours at 18º C.

EQUIPMENT AND MATERIALS:

Equipment:
Binocular microscope with 10X, 20X, or 40X objectives.
Incubator.

Materials:
Disposable gloves used in handling specimen and inoculating the test.
Disposable pipettes for inoculation of urine sediment.
Sterile individually wrapped cotton swab.
Small biohazard bags

Preparation: N/A

Storage Requirements:
Store the InPouch TV test kits at room temperature, 18-25º C. Temperatures greater than 25º C are NOT RECOMMENDED and may damage the product. Product shelf life is 12 months from date of manufacture. Although the plastic film of the pouch is resistant to water vapor loss, some may occur over time. Pouches should be stored vertically, in their closed box. The medium should not be refrigerated or frozen.

An InPouch containing a cloudy medium or precipitate should not be used.

CALIBRATION: N/A

QUALITY CONTROL:

The Biomed InPouch TV diagnostic medium is manufacture in small lots. Each lot is released only after a QC test of viability, doubling time, and purity. If an active trichomonad culture is to be maintained, inoculate a new pouch with on to two drops (30ul) of the actively growing culture and incubate at 37º C for 24 to 48 hours with subsequent holding at 32º C. The subculture will be viable for two to four days, depending on the isolate. Sub-culturing should be done with the organism each a concentration of 1 x 10^4 per ml. A secondary product, the Subculture Medium, Catalog No: 10-2108, can be used to maintain a culture for up to seven days.

PROCEDURE - STEPWISE:

1. Inoculation. Remove InPouch TV pouch from box. Fold pouch back over itself to reduce the folded crease. Make sure that the liquid in the upper chamber is below the closure tape to prevent fluid from leaking upon opening. Tear open the pouch at the notch just above the closure. Open the pouch sufficiently to admit the swab by pulling the closure tape's middle tabs apart. Insert swab containing the specimen into the liquid of the pouch's upper chamber. Squeeze sample from the swab by gently pressing it between the walls of the upper chamber, **discard the swab in a biohazard container**. If urine sediment is being inoculated (~1-2 drops), the sediment can be inoculated into the pouch by using a sterile disposable pipette. The production of bubbles in the inoculation process should be minimized. **In no immediate microscopic examination is desired, go to procedure #4.**
2. **Immediate microscopic evaluation (wet mount**). Squeeze pouch closed, fold the top edge down and roll 3-4 times. Fold the wire tape's end-tabs behind the pouch to lock the roll. Place the upper chamber of the pouch on the raised platform of the open microscope clip. Close and lock the clip over the pouch. The pouch should be viewed from the upper (open winder) side of the microscope clip. Observe with a microscope under low power (10x or 20x). Use high dry (40x) power if necessary for confirmation. When no trichomonads can be found in the upper pouch chamber. The inoculum has less than 100 organisms. Remove microscope clip after observation.
3. **Immediate specimen concentration**. It is possible to concentrate the cellular material by standing the pouch vertically for at least 15 minutes prior to microscopic evaluation. The trichomonads will concentrate at the bottom of the chamber. The microscope clip is then placed as described in Procedure #2 listed above. Observe for motile organisms.
4. **Culture and microscopic evaluation**. Express the contents of the upper pouch chamber into the lower chamber. Toll down the pouch until the tape is at the top of the label. Fold the wire tapes' end tabs to lock the roll. This action helps maintain partial anaerobiasis. Incubate the pouch vertically at 37º C for 18-24 hours. Prior to reading, pull the pouch upward (approximately 5 times) across the edge of a table for mixing.

Place the bottom of the lower chamber on the raised platform of the open microscope clip, then close and lock the clip over the pouch. Observe microscopically under low power (10x). The best location in the pouch to find trichomonads is slightly above the bottom edge of the pouch. **Repeat evaluations daily for the presence of motile trichomonads for up to 5 days.** Do not mistake Brownian motion of small debris particles for evidence of trichomonad activity.

CALCULATIONS: N/A

REPORTING RESULTS:

Trichomonas vaginalis present: Confirming the presence of motile trichomonads after wet mount, concentration, or after 1 to 5 days of culture using microscopic evaluation.

No *Trichomonas vaginalis* found: Reported as a preliminary report if wet mount and concentration observations are negative; as a final report, if no trichomonads are found after incubating the InPouch test over a 5-day period.

Procedures for Abnormal Results:

If an abundance of debris makes viewing difficult, record this observation when reporting for each specimen collected.

Reporting Format:

For each specimen collected, record the specimen identification number, date of observation and a positive (P or +) if trichomonads are observed and a negative (N or -) if no trichomonads are observed. If a specimen is positive, after recording the result, discard the specimen in a biohazard waste container. However, if a specimen is negative, return the specimen to the incubator and evaluate again after 24 hours (up to 5 days). Additionally, record any abnormal observations for the specimen identification number in the comments column.

PROCEDURE NOTES:

Specificity: The InPouch is for cultivation of *T. vagnialis* only. Other Trichomonas species will not survive and replicate at the PH and media composition found in the InPouch test kit.
Culture: *T. vaginalis* replicates within several hours after inoculation into the InPouch test. An inoculum containing 1 to 10 organisms is sufficient to cause a positive test.
Reading tip: Because a large volume of fluid is being scanned, more canning of the volume of medium is required when examining the InPouch TV in comparison to a wet-mount preparation. Make sure to focus in the liquid and not the textured plastic of the pouch.

LIMITATIONS OF THE PROCEDURE:

Decreased recovery will occur if patient specimens are contaminated with birth control foams or jellies. WARNING: This product contains a chemical known to the State of California to cause cancer, birth defects or other reproductive harm.

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CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date: 1/Sept/2005 Author: Physiwell M Sikateyo, Jennifer Westerman
Title: Uristix 4 Multistix for Dipstick Urinalysis		Revised Effective Date: NA Author: NA
Proc#:	File Name: Urinalysis SOP Bayer	

I. PURPOSE/PRINCIPLE: To provide a procedure for the use of the Bayer reagent strips for urinalysis. The reagent strips provide tests for glucose, protein, nitrite and leukocytes in urine. Refer to package insert for exact chemical principle.

II. MATERIALS AND EQUIPMENT:

Reagent strips
Urine specimens
Chek-Stix (Positive and Negative Control)
Timer

1. Store the reagent strips at room temperature, between 15-30 degrees Celsius, away from direct sunlight.
2. If the testing area is above 30^{o}C, transfer individual test strips to the testing area within an hour of use, keeping the strips in a light-proof container from the time of transfer until used for testing.
3. Never use the product once it expires.

III. SECIMEN TYPE/STORAGE:

A. Urine – first stream "unclean catch". See Collection SOP for collection instructions.
   1. If testing can not be done one hour after voiding, refrigerate the specimen immediately.
   2. Allow the urine to return to room temperature before testing.

IV. FORMS:

Beyer Uristix 4 QC Form
Clinical Examination Report

V. PROCEDURE:

A. <u>Urine Testing</u>

1. Mix urine sample well before testing.

2. Remove one strip from the bottle and immediately replace the cap. Completely immerse reagent strip areas in fresh urine and remove immediately to avoid dissolving out reagents.
3. While removing, run the edge of the entire length of strip against the rim of the urine container to remove excess urine.
4. Hold the strip in a horizontal position to prevent possible mixing of chemicals from adjacent reagent areas and/or contaminating the hands with urine
5. Visually compare the reagent areas to the corresponding color chart on the bottle label at the time specified. Hold strip close to color blocks and match carefully.
6. Avoid laying the strip directly on the color chart as this will result in the urine soiling the chart.
7. Proper read time is critical for optimal results.
   a. **Glucose** read test at 30 seconds after dipping.
   b. **Protein** and **nitrite** tests read at 60seconds after dipping.
   c. **Leukocytes** test read at 2 minutes after dipping.
8. Color changes that occur after stipulated time are of no diagnostic value.

B. QUALITY CONTROL: Performed by CIDRZ Central Laboratory

1. Prepare QC samples:
   a. Label two tubes for controls, positive "+ve" and negative "-ve".
   b. Place 12 mls of deionized water in the labeled tubes. Do NOT use tap water.
   c. Remove a Chek-Stix control from the bottle and immediately replace the cap tightly.
   d. Place the strip in the tube and cap tightly.
   e. Repeat steps a. to d. for the second control.
   f. Set timer for two minutes.
   g. Gently invert the tubes back and forth for two minutes.
   h. Allow the tubes to stand for 30 minutes at room temperature. Set timer.
   i. Invert one more time, then remove and discard the strips.
   j. After reconstitution, control solutions are treated as a urine specimen.
2. Perform QC:
   a. QC of **each** bottle is performed at the CIDRZ central laboratory prior to distribution of reagent sticks.
   b. Performance of reagent strips is confirmed by testing known negatives and positive specimens or controls whenever a new bottle is first opened.
   c. Write "opened" and the date on the bottle when it is opened.
   d. Perform the test using the positive and negative controls.
   e. Document the Test and QC information on the Bayer Uristix 4 QC Log.
   f. Enter the results of each control tested on the log.
   g. Compare results of controls to the values in the following chart.
   h. Document if the control passes or fails.

QC Values

Test	Positive Control	Negative Control
Glucose	100 mg/dl-250 mg/dl	Negative
Protein	Trace – 100 mg/dl	Negative
Nitrite	Positive	Negative
Leukocytes	Trace - Moderate	Negative

i. Perform the following actions if the controls fail.
   1) Make up new control.
   2) Retest.
   3) Document repeat on the Bayer Uristix 4 QC log.
j. Results are reviewed prior to dispatch to the clinics.
k. Write date of dispatch and the name of the clinic to which the bottles have been sent.
l. File the QC into the appropriate box file.

VI. RESULTS/REPORTING:

A. Results with Bayer Reagent Strips are obtained in clinically meaningful units directly from the color chart comparison when using strips visually.
B. QC results are reviewed by the supervisor/designee prior to dispatch to the clinics.
C. Write the name of the clinic and the date of dispatch on the Bayer Uristix 4 QC log.
D. File the QC log in the Reagent/Kit QC box file.

VII. LAB REFERENCE RANGE: NA

VIII. LIMITATIONS:

- Prolonged exposure of urine to room temperature may result in microbial proliferation with resultant changes in pH
- A shift to alkaline pH may cause false positive results with the protein test area.
- Glucose concentration may also decrease as the bacteria metabolize the glucose.
- Nitrite test results are optimized by using a first morning specimens or one which has incubated in the bladder for four hours or more. In random urine specimens from females, positive results for leukocytes may be due to a source external to the urinary tract.
- Contamination of the urine with specimen with the cleansers containing chlorhexidine may affect protein test results.

IX. SPECIAL NOTES:

- Urinalysis dipstick test performed by nurses in the clinics at the patient's bedside.

X. REFERENCES:

Package insert. Bayer Uristix 4-Multstix 2 Reagent strip for urinalysis manufactured by Bayer Corporation, Elkhart, IN USA.

CIDRZ – Central Laboratory PO Box 34681 Kalingalinga Clinic Lusaka, Zambia STANDARD OPERATING PROCEDURES		Originating Effective Date: March 2001 Author: P. Sikateyo, P. Chipaila, A. Taylor
Title: Urine hCG Pregnancy Testing – Quidel QuickVue		Revised Effective Date: Author: P. Sikateyo, J. Westerman
Proc#: V.1.1-4	File Name: V.1.1-4 Urine hCG Pregnancy Testing - QuickVue	Page 531 of 538

I. PRINCIPLE/PURPOSE: To provide a procedure for performing a qualitative hCG assay using the QuickVue One Step hCG urine test. The Quidel QuickVue test uses a monoclonal antibody to the beta subunit of hCG to detect hCG in a single-step technology.

II. MATERIALS AND EQUIPMENT:

Quidel Quick Vue one-step hCG test kit with test cassettes
Disposable pipettes (supplied with the test kit)
Clock
Timer or stop watch
Quidel hCG Control Set (CN 00272)

III. SPECIMEN TYPE/STORAGE:

A. Specimen Type

1. Urine is the specimen used in the test.
2. First morning specimens generally contain the highest concentration of hCG and are recommended for early detection of pregnancy.
3. If a first morning specimen is not possible it is recommended that **voiding not occur within one hour** of providing the urine specimen for hCG pregnancy testing.
4. Refer to the protocol collection SOP for collection details.

B. Specimen Storage

1. Specimens may be kept at room temperature (15 to 30°C) for 8 hours or refrigerated at 2-8 °C for up to 72 hours.
2. Samples may be frozen once at - 20 °C or below. If frozen, mix after thawing. Do not refreeze.

C. Kit Storage

1. Store the test cassette/kits at room temperature (15-30 °C) away from direct sunlight. Cassettes/Kits are stored in the Satellite Laboratory.
2. The cassettes/kits are stable until the expiration date.

3. hCG Control Set is stored at room temperature (15-30 °C) in the Satellite Laboratory until expiry date.
4. The room temperature at which testing is performed must be 15-30 °C.

IV. FORMS:

V.1.1A Urine hCG Quality Control Bench Log
V.1.1B Urine hCG Pregnancy Testing Bench Log
Protocol Clinical Examination Room Form (CER)

V. PROCEDURE:

A. Performing Test

1. Verify the expiry date and Lot Number of the QuickVue test cassette with those listed on the Urine hCG Pregnancy Testing Bench Log. (If the cassette information is different raise a new Urine hCG Pregnancy Testing Bench Log).
2. Verify the temperature of the room is 15 to 30 °C.
3. Remove the QuickVue test cassette from the foil pouch and label it with the participant ID.
4. Place the test cassette on a clean, dry, level surface.
5. Set timer for 3 minute countdown.
6. Using the disposable pipette supplied, pipette urine and add **3 drops** (125µl) of urine to the **Round Sample Well** on the test cassette.
7. Start timer and look at the clock. **Write the test start time** on the **Source Document**.
   Central Lab Note: If performing more than one test, use a new pipette for each sample/test.
4. The test cassette should not be handled or moved until the test is complete and ready for reading.
5. After the timer beeps (3 minutes) **read** result.
6. Look at the clock time and **write the test read time** on the **Source Document**.
7. Write the test result on the **Source Document**. Refer to section **VI. Result/Reporting**.

B. Documenting Test Results – V.1.1B Urine hCG Pregnancy Testing Bench Log

1. Write the Manufacturer, cassette/kit Lot Number, expiry date and the clinic location on the top of the form if a new Lot Number is being used, otherwise verify that kit Lot Number and expiry date matches what is already at the top of the form and place a tick ✓.
2. Write the date of testing.
3. Write the patient or participant ID number.
4. Write the test start time and test read time.

5. Write the participant's test results.
6. If test is invalid, write in Comments/Action the action performed. For example, write "test repeated using different cassette".
7. Write your initials and code, if applicable.

C. Quality Control

1. **External Quality Control-** performed by the study nurses.
   a. Positive and Negative test result control solutions are used:
      1) Once per week at the beginning of the week.
      2) Prior to using a new Lot Number of test kits.
   b. One study nurse will perform the Quality Control testing each week. Study nurses will rotate weekly to perform Quality Control.
   c. Document the quality control results on **V.1.1A Urine hCG Quality Control Bench Log**.
2. **Internal** (Built-into the test cassette) **Quality Control**
   a. The appearance of a **blue Control Line** indicates that the correct amount of urine was added.
   b. The background of the reading window of the test cassette should be **white to light pink** within 3 minutes of urine addition and should not interfere with easy reading of the test result.
   c. Indicate whether the **Blue Control Line** was present or not by circling **Yes** or **No** on **V.1.1A Urine hCG Quality Control Bench Log**.

   Note: If this line does not develop, the test result is considered invalid – **QC FAILS**.
3. Actions for Failed QC:
   a. Repeat testing using a different lot number of positive and negative controls.
   b. If a different control lot number is **not available**, test the urine from a pregnant woman (early in her pregnancy). If the test is negative a new lot number of cassettes are required.
   c. Notify the laboratory supervisor or management staff.
   d. **Do not perform testing for participant's until quality control is resolved.**
   e. Satellite lab tech will use Corrective action form to document problem and actions.
4. **Successful Quality Control – QC PASS**:
   a. Blue Control line is present.
   b. Background is white to light pink.

VI. RESULT/REPORTING:

A. Test Interpretation

1. **POSITIVE:** A blue control line (C) and any pink-to-red Test line (T) is a positive result for the detection of hCG.
2. **NEGATIVE:** A blue control line (C) and no pink Test line (T) is a negative result.
3. **INVALID RESULT:** No blue control line is visible at 3 minutes. If this happens, retest using a new test cassette.

Note: If repeat test is invalid repeat positive and negative controls.

B. Reporting

1. Test results are recorded on the **Source Document**.
2. Test results are also written on **V.1.1B Urine hCG Pregnancy Testing Bench Log**.
3. **VI.1.1A Urine hCG Quality Control Bench Logs** are reviewed monthly by the QA/QC Coordinator, Lab Study Coordinator or designee.
4. **VI.1.1B Urine hCG Pregnancy Testing Bench Logs** are reviewed monthly by the QA/QC Coordinator, Lab Study Coordinator, Quality Control Nurse or designee to confirm performance of the quality control.

VII. LAB REFERENCE RANGE:

- Specimens containing as low as 25 mIU/ml hCG will yield positive results.

VIII. LIMITATIONS:

- Early pregnancy associated with a low level of hCG, may show color development after 3 minutes procedure time. If a negative result is obtained, but pregnancy is suspected, another specimen should be collected after 48 – 72 hours and tested. Document in the comment section on the pregnancy testing bench log.

- While pregnancy is the mostly likely reason for the presence of hCG in urine, elevated hCG concentrations unrelated to pregnancy have been reported in some women with conditions such as ectopic pregnancy, molar pregnancy, trophoblastic and nontrophoblastic diseases.

- hCG may remain detectable for a few days to weeks after delivery, abortion, natural termination or hCG injections.

IX. SPECIAL NOTES:

- Turn around time for results (TAT) = results same day of collection.
- The **Source Document** is defined by each protocol/study.
- A copy of the document is maintained in the SRN's office in clinics where testing is performed by study nurses.

X. REFERENCES:

Package Insert. Quidel QuickVue one step hCG Urine Test. Quidel Corporation. San Diego, CA USA.

Package Insert. Quidel hCG Control Set. Quidel Corporation. San Diego, CA USA.

Satellite Laboratory
Weekly Quality hCG Control Bench Log

Kit Lot #: _____ Kit Expiry Date: _____

Date	Pos Control Lot #	Pos Control Expiry Date	Pos. Control Results	Neg Control Lot #	Neg Control Expiry Date	Neg. Control Results	Control Line Y/N	Background Acceptable* Y/N	Pass/Fail	Comments	Tech 01	Tech 02

*Acceptable Background: White to light pink

Section 17: Other Laboratory Issues

Some studies may involve urine drug of abuse testing (DOA). DOA requires extra attention to specimen collection to prevent introduction of tampered or other-person specimens. Due to the ELISA methodology involved in many of the rapid testing kits, positive results will be indicated by the absence of a line and a negative result will be indicated by the presence of a line. This is the opposite of most rapid tests such as HIV or hCG. Extra attention should be paid to test reading, reviewing and reporting to avoid erroneous results.

Some studies may require PAP smears. These may be performed on site or the slides may be shipped for external reading. PAP smear processing requires cytology and pathology support and which is not addressed in depth in this document.

Section 18: Useful Internet References

Disclaimer: These links are provided as useful references. These web addresses are current at the time of this release but may change. The information in these links is not guaranteed or endorsed by HPTN or MTN except for those listed under network sites.

Specimen Shipping, Shipping Materials and Information:

WHO Transport of Infectious Substances	http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2005_22/en/
CDC Shipping Regulations	www.cdc.gov/od/ohs/biosfty/shipregs.htm
Code of Federal Regulations	http://www.gpoaccess.gov/cfr/index.html
US Department of Transportation	http://www.dot.gov/
US Postal Service	http://www.usps.com
Saf-T-Pak	http://www.saftpak.com
IATA	http://www.iata.org/index.htm
CDC Biohazard Policy	http://www.cdc.gov/od/ohs/biosfty/biosfty.htm

Risk Group Assessments:

NIH Appendix B	http://www4.od.nih.gov/oba/rac/guidelines%5F02/appendix%5Fb.htm
American Biological Safety Association	http://www.absa.org/XriskgroupsX/index.html
CDC Select Agent Program	http://www.cdc.gov/od/sap/
USDA Plant and Animal Pathogen Select Agent Program	http://www.aphis.usda.gov/programs/ag_selectagent/index.html

Monitoring sites:

US Federal Drug Administration	http://www.fda.gov/
LDMS	http://www.fstrf.org/ldms/
College of American Pathologists	http://www.cap.org/apps/cap.portal?_nfpb=true&_pageLabel=home
UK National External Quality Assessment Service	http://www.ukneqas.org.uk/
PPD	http://www.ppdi.com/
pSMILE	http://162.129.103.142/SMILE/

Network sites:

MTN	http://mtnstopshiv.org
HIV Preventions Trial Network (HPTN)	http://www.hptn.org/index.htm
HIV/AIDS Network Coordination (HANC)	http://www.studysource.org/

HPTN Requirements, Frequently Asked Questions (FAQs)	http://www.hptn.org/hptn_structure/CentraLab/HIVABTestReqs.htm